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AUTONOMIC NERVOUS CONTROL
OF
CEREBROSPINAL FLUID PRODUCTION
FROM
THE CHOROID PLEXUS

BY

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Fluorescence Histochemical Study on Regional Differences in the Sympathetic Nerve
Supply of the Choroid Plexus from Various Laboratory Animals

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Summary. The adrenergic nerve supply of the choroid plexus from all four ventricles was studied by the Falck-Hillarp histofluorescence technique from nine different species of animals, and the noradrenaline concentration in whole plexus tissue was determined by a radioenzymatic method. The nerve density was usually in the order third > lateral > fourth ventricular plexuses. Pig and cat plexuses had the largest number of nerves, the innervation was intermediary in those from baboon, guinea-pig, rat, rabbit and hamster, whereas only few fluorescent nerves were found in cow and mouse plexuses. Sympathetic denervation showed ipsilateral supply from the superior cervical ganglia to the lateral plexuses and a mixed contribution to the midline ones. The total noradrenaline concentration varied between 0.10 and 0.73 ng per mg protein.

Key words: Choroid plexus, laboratory animals — Sympathetic nerves — Fluorescence histochemistry, catecholamines — Biochemical quantification, noradrenaline.

The mammalian choroid plexus receives sympathetic adrenergic nerves whose terminals run in association both to blood vessels and the plexus epithelium (Edvinsson et al., 1974). Electron microscopy has shown that these terminals form close contacts with the smooth musculature of small arterioles as well as with the epithelial cells (Edvinsson et al., 1975). There is strong evidence for an inhibitory influence of the sympathetic innervation on the cerebrospinal fluid production (Edvinsson et al., 1975; Lindvall et al., 1978a and b). Also cholinesterase-containing nerves have been shown in the mammalian choroid plexus at the light microscopic level (Edvinsson et al., 1973; Lindvall et al., 1977), and ultra-structurally non-adrenergic, probably cholinergic, nerve terminals form close contacts with the epithelium as well as with the arterioles (Edvinsson et al., 1975). In a comparative histochemical study on regional differences in the cholinergic nerve supply of the choroid plexus from ten different laboratory animals a positive acetylcholinesterase staining was found in all except the hamster (Lindvall et al., 1977). Usually, the plexus of the third ventricle was best supplied, whereas plexuses of the fourth ventricle received comparatively few nerve fibres. The most well-developed nerve supply was found in plexus tissue from pig and cat (Lindvall et al., 1977).

The present study was undertaken to give a more detailed account of the adrenergic nerve supply of choroid plexuses of different animals, including regional variations, and to some extent compare the species variations in the adrenergic innervation with the cholinergic supply. The histochemical findings were correlated with biochemical estimation of the noradrenaline content in the tissue.

Materials and Methods

Animals. Choroid plexuses from all four ventricles were studied in nine different species as listed in Fig. 2. Unilateral sympathectomy was performed in 6 rabbits by removal of the superior cervical ganglion on either the right or left side under nembutal-ether anesthesia and the animals were killed 2 weeks later.

The bovine and porcine plexuses were obtained about 15 min after the animals had been killed at a local slaughter-house. The other animals were killed by exsanguination or decapitation under light ether or nembutal anesthesia. The plexuses were immediately dissected out and further processed for fluorescence microscopy and biochemical catecholamine determinations.

Fluorescence Histochemistry. The plexuses were treated in two different ways. They were prepared either as whole-mounts, which were gently spread on microscope slides, dried

for 1 hr at room temperature in a desiccator over phosphorous pentoxide, and exposed for 1 hr to formaldehyde gas at $+80^{\circ}\text{C}$ and optimal humidity according to the Falck-Hillarp histofluorescence technique (for details, see e.g. Björklund et al., 1972). Or, the plexuses were frozen to the temperature of liquid nitrogen in a propane-propylene mixture, freeze-dried, treated with formaldehyde vapour as above, paraffin-embedded in vacuo, and sectioned serially at $5\text{ }\mu$ thickness (Björklund et al., 1972). The whole-mounts and paraffin sections were mounted in Entellan (Merck) and analyzed in a Zeiss fluorescence microscope equipped with a BG 12 primary and 47 + 50 secondary filters (Björklund et al., 1972).

Biochemical Determinations. Plexuses from the lateral, third, and fourth ventricles were pooled from 1 - 20 animals of each species, immediately frozen in liquid nitrogen and stored in a deep-freezer at -65°C until assay. The samples, weighing 10 - 800 mg, were homogenized in 0.1 N perchloric acid to which 10 mg % EDTA was added. After centrifugation the supernatant was taken for catecholamine assay according to the catechol-O-methyl transferase method described by Cuellar et al. (1973), but modified to use a high specific activity ^3H -methyl-S-adenosyl methionine and ethyl acetate extraction (Coyle and Henry, 1973). Tissue protein was determined according to Lowry et al. (1951).

Results

The adrenergic nerves were arranged in well-defined networks around the major blood vessels supplying the plexuses. Delicate varicose terminals also formed networks in the stroma among the epithelium or ran as isolated fibres, sometimes along small blood vessels (Fig. 1).

In the fluorescence microscope clear species differences were observed with regard to the density of nerves per unit area of plexus (Fig. 2). The choroid plexuses of pig (Fig. 1 a) and cat (Fig. 1 b) were most well-supplied with nerves both to the vasculature and epithelium; baboon (Fig. 1 c), guinea-pig, rat (Fig. 1 d), rabbit and hamster had an innervation of intermediary density, while the plexuses of cow (Fig. 1 e) and mouse received comparably few adrenergic nerve fibres. In pig and guinea-pig the adrenergic nerve supply of blood vessels predominated as compared to the plexus tissue proper with its epithelium, while the opposite relation was found for the cat and rat. In the plexus tissue from cow there was a striking presence of green-fluorescent mast cells, which were concentrated especially around those vessels not receiving visible adrenergic nerves (Fig. 1 e). The nerve density was in the order third > lateral > fourth ventricular plexus.

Unilateral removal of the superior cervical ganglion on either the left or right side was carried out on rabbits. Fluorescence microscopy showed a complete disappearance of nerves in the ipsilateral plexus from each lateral ventricle. The number of fluorescent nerves in the plexus tissue from the third and fourth ventricles was markedly reduced.

The radioenzymatic measurements of noradrenaline in plexus tissue pooled from all four ventricles showed the presence of measurable amounts of catecholamines in all species examined. The mean values (expressed as ng amine per mg protein in the entire plexus tissue) from two aliquots of pooled tissue or two separate determinations on unpooled tissue were (number of animals within parenthesis): pig (3), 0.15; cat (3), 0.30; baboon (2), 0.73; guinea-pig (11), 0.73; rat (12), 0.13; rabbit (3), 0.34; cow (2), 0.10; mouse (19), 0.53; hamster (9), 0.55.

Discussion

The formaldehyde method of Falck and Hillarp (for details, see e.g. Björklund et al., 1972) which was used in the present study provides a highly sensitive and specific way to demonstrate catecholamine-containing neurons in the fluorescence microscope. Fibre structures with a specific fluorescence of green colour under the optical conditions used together with chemically measurable noradrenaline was taken as evidence for the presence of noradrenergic nerves in the plexus tissue. The radiometric-enzymatic assay has a high sensitivity for measuring amines in small amounts of tissue and in low concentrations (Cuello et al., 1973; Coyle and Henry, 1973) and it is therefore well suited for the present type of studies, involving small tissue samples. Noradrenaline and adrenaline cannot be differentiated by this procedure, but there is not any marked cross-reaction between them and the catechol precursors of noradrenaline (Coyle and Henry, 1973). The conditions under which the fluorophore was formed (Björklund et al., 1972) supported that the nerve fluorescence represented noradrenaline.

In all animal species the innervation pattern was similar, with terminals coming in close contact with both small blood vessels (arterial as well as venous) and the plexus epithelium. The adrenergic nerve terminals formed networks around the blood vessels and they also ran as isolated fibres in between the vessel wall and the overlying epithelium. This pattern agrees with earlier studies (Edvinsson et al., 1974; Lindvall et al., 1978a) and it also resembles the pattern of the cholinergic innervation demonstrated by acetylcholinesterase-staining (Edvinsson et al., 1973; Lindvall et al., 1977).

There was a distinct species difference in the amount of plexus innervation. The species variation in the innervation density of adrenergic nerves did not in all parts correspond to that of cholinergic nerves (Lindvall et al., 1977). In the rat, for example, the adrenergic nerves were more numerous than the cholinergic ones, while the contrary was seen in choroid plexuses of cow. The choroid plexus of the rabbit has earlier been shown to contain measurable amounts of noradrenaline by fluorometric and radioenzymatic techniques (Edvinsson et al., 1972; Lindvall et al., 1978a; Lindvall and Owman, 1978). In this study, noradrenaline could be demonstrated in all animal species investigated with concentrations varying between 0.10 and 0.73 ng/mg protein. The lack of a clear-cut agreement between the histochemical and quantitative findings may be explained, at least partly, by the presence of varying amounts of blood influencing the protein content in the tissue from the different species. No attempt was made to perfuse the tissue to overcome this, because perfusion would for practical reasons not have been feasible in all instances.

As a general feature among the different animal species, the adrenergic innervation predominated in the plexus of the third ventricle, the lateral ventricles being somewhat less innervated, while the plexus of the fourth ventricle received the fewest fibres. The tissue level of noradrenaline in the different plexuses of adult rabbit showed a corresponding ratio, with a noradrenaline content of 1.46, 0.27 and 0.15 ng amine/mg protein in the plexus of the third, lateral and fourth ventricle, respectively (Lindvall and Owman, 1978).

The adrenergic fibres arise principally from the superior cervical sympathetic ganglia as revealed in denervation experiments on cats, rats and rabbits (Edvinsson et al., 1972, 1974, and 1975; Lindvall et al., 1978a). There is, however, evidence that part of the sympathetic innervation in the rabbit originates from more caudally located ganglia than the superior cervicals (Edvinsson et al., 1975; Lindvall et al., 1978a). Unilateral denervation (carried out on rabbits) showed a strictly unilateral distribution of the superior cervical ganglia in the lateral plexuses, whereas nerves from both sides mixed in the mid-line plexuses.

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Legends to illustrations

- Fig. 1. Examples of the adrenergic nerve supply of the choroid plexus taken from various animal species as demonstrated by the Falck-Hillarp histofluorescence technique on whole-mounts or paraffin sections. (a) Pig (lateral ventricle), X 270; (b) cat (third ventricle), X 180; (c) baboon (lateral ventricle), X 180; (d) rat (third ventricle), X 190; cow (third ventricle) containing both green-fluorescent mast cells and nerve fibres, X 190.
- Fig. 2. Summary of adrenergic nerve supply estimated by fluorescence microscopy of choroid plexuses from the various animal species. Arbitrary units from 1 (scattered nerves) to 6 (extensive supply). The ventricles from which the plexuses were removed are identified to the right, with the 1-unit symbol. Number of animals is indicated within parentheses. Corresponding illustrations from the microscopic analyses are found in Fig. 1.



Fig. 1

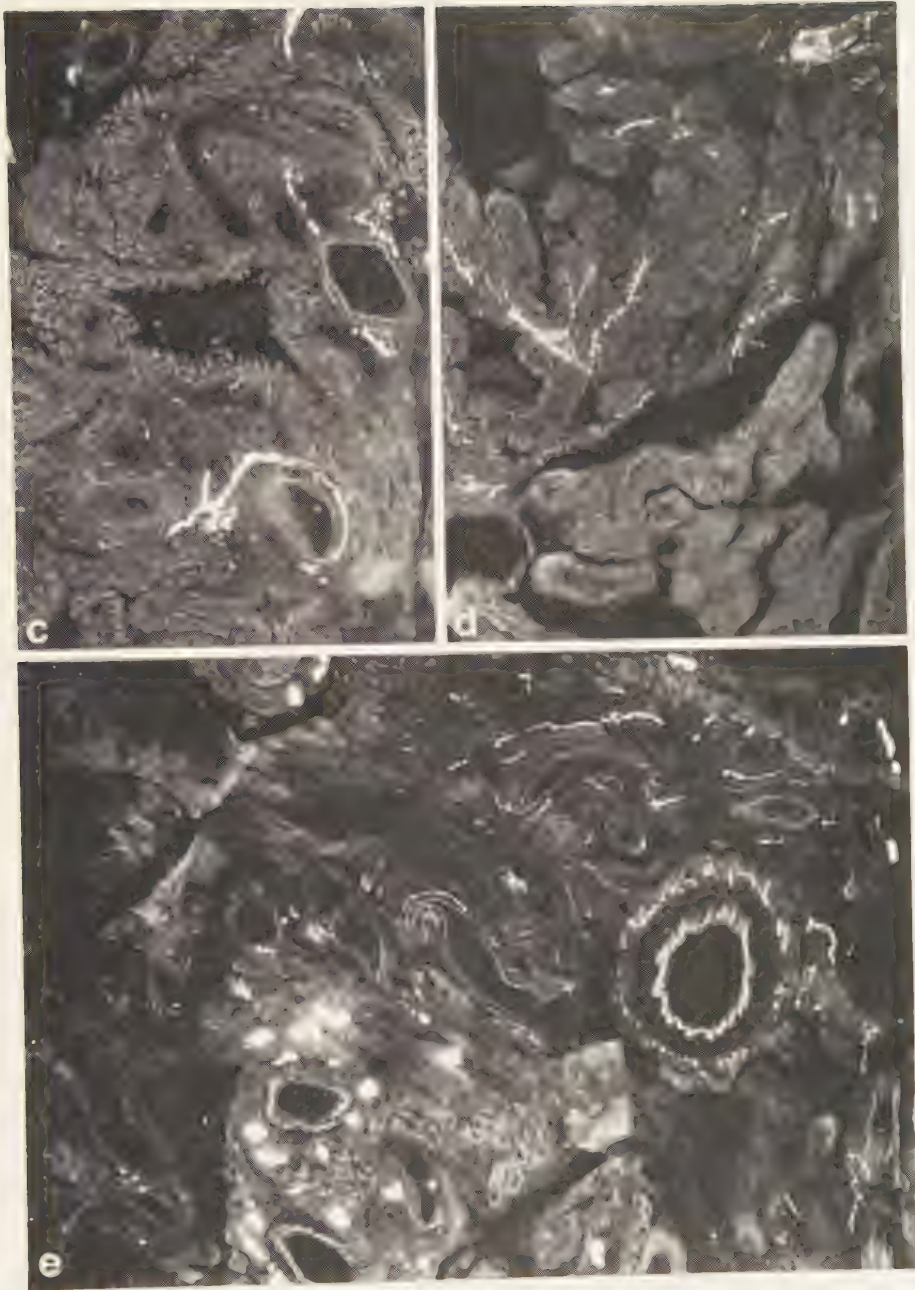


Fig. 1

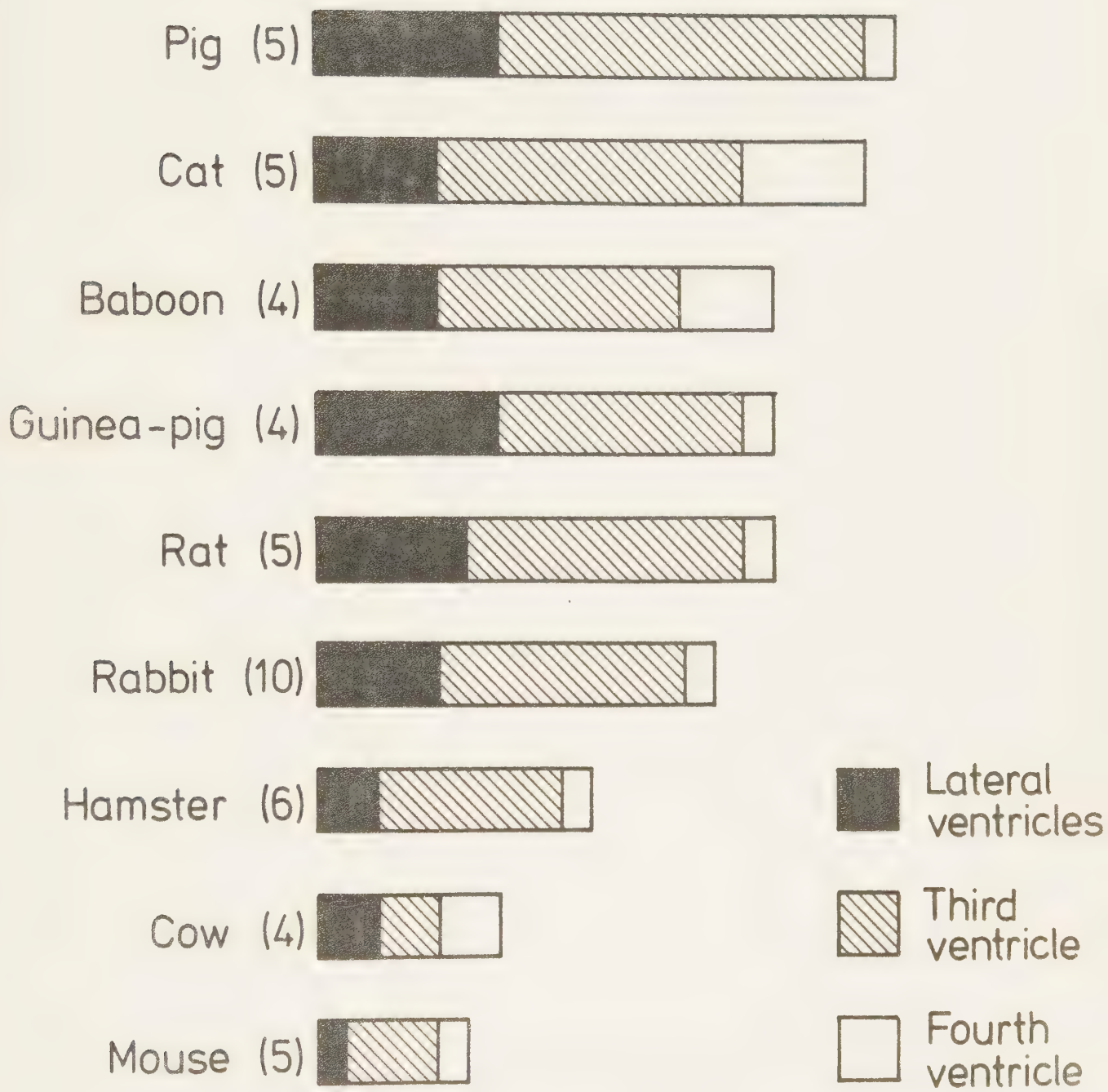


Fig. 2

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Effect of Sympathomimetic Drugs and Corresponding Receptor
Antagonists on the Rate of Cerebrospinal Fluid Production

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Running title: Sympathomimetic effects on CSF production

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(Abstract)

The bulk production of CSF was measured in rabbits on the basis of ventriculocisternal perfusion and dilution of ^{14}C -inulin. Intraventricular or i.v. infusion of norepinephrine produced a dose-related decrease in the production by as much as 50%, the effect being counteracted by both α - and β -antagonists (except when the latter was given i.v., which potentiated the i.v. norepinephrine response). Also intraventricular (but not i.v.) administration of the β_1 -receptor agonist, H80/62, reduced CSF formation (effect blocked by practolol), in contrast to the β_2 agonist, terbutaline, which had little or no effect. It is suggested that the sympathomimetic reduction in the rate of CSF formation is the result of a combined β_1 -receptor mediated inhibition of the secretion from the plexus epithelium and a reduced blood flow in the tissue resulting from stimulation of the vascular α -receptors.

INTRODUCTION

Electrical stimulation of the sympathetic trunks in the neck results in a marked reduction in the net rate of cerebrospinal fluid (CSF) production as measured in rabbits (16) by the ventriculocisternal perfusion technique of Pappenheimer and co-workers (23) based on radioactive inulin dilution (13). The effect of stimulation is probably mediated directly through an action on the choroid plexus, which has a well-developed supply of adrenergic nerve terminals originating in the superior cervical sympathetic ganglia (11). In agreement with this, bilateral removal of these ganglia, which reduces the concentration of norepinephrine in the plexus tissue to insignificant levels, results in a marked increase in the CSF formation (16). The distribution of the adrenergic nerves in close relation both to the plexus epithelial cells and its blood vessels (8) suggests that the altered production rate is the consequence of influences not only on plexus blood flow but also on the secretory cells. This is supported by observations showing that sympathetic denervation of the plexus enhances the carbonic anhydrase activity in the choroid plexus (8). Moreover, the effect of sympathetic nerve stimulation on plexus blood flow (of rabbits) appears to be only slight (A. Bill, personal communication).

The present study was undertaken to investigate the nature and distribution of the local adrenoceptors mediating the sympathetic nerve-induced effect on CSF formation by infusion of sympathomimetic agonists and corresponding specific receptor antagonists via the intraventricular or vascular routes during registration of the ^{14}C -inulin dilution in the ventricular system of rabbits.

MATERIAL AND METHODS

Experimental. The procedure for quantitative measurement of the net rate of CSF formation in rabbits was used largely as described by Aquilonius and Winbladh (1), which is a modification of the original method developed by Pappenheimer et al. (13, 23).

Experiments were performed on 137 albinic and randomly pigmented rabbits of either sex, weighing 2.5 – 3.5 kg and maintained on standard pellet food and tap water ad lib.

The animal was initially lightly anesthetized with nembutal (20 mg/kg) slowly into the ear vein. Further anesthesia was instituted in an open system with 2.5 – 3 % halo-

thane in dinitrous oxide and oxygen (2:1) using a mask. The animal was tracheotomized through a midline incision in the neck. A T-tube was inserted and connected with a respirator; the halothane concentration was from now on reduced to 0.5 %. The animal was paralyzed with 0.1 – 1 mg/kg d-tubocurarine chloride i.v.; additional doses were given as needed. Heparinized teflon catheters were placed in direction towards the aorta via the femoral arteries (one for blood pressure measurements by a Statham P23AC pressure transducer, the other for sampling in the blood gas measurements). Catheters were also introduced into one or both femoral veins for the i.v. injections and infusions.

The animal was placed in prone position in a specially made hammock and the head fixed in a metal frame. Through a burr hole in the skull a small cannula (22), occupying an intracranial volume of 0.0093 cm^3 (10), was inserted into the right ventricle of the brain and fixed by a rubber plug. The atlanto-occipital membrane was dissected free for insertion of an outflow needle (0.8 mm o.d.) which was fixed to an adjustable level in the head frame. The body temperature of the animal was maintained normal with a heating blanket and was checked by a thermometer inserted into the rectum. Shortly before perfusion was started, the halothane was excluded from the anesthetic mixture, and the animal was in the following anesthetized only with $\text{N}_2\text{O}/\text{O}_2$.

Artificial CSF, warmed to 38°C , was infused by an infusion pump (Braun, Melsungen) through the inflow probe at a constant rate of $30 \mu\text{l}/\text{min}$. The CSF buffer solution had the following composition (mM): NaCl 123, CaCl_2 0.86, KCl 3.0, MgCl_2 0.89, NaHCO_3 25, NaH_2PO_4 0.50, Na_2HPO_4 0.25; it was previously gassed with 5 % CO_2 in O_2 (pH 7.20 – 7.30). The inflow catheter was connected via a T-line to a Statham Model P23AC pressure transducer for recording of infusion pressure. Drainage through the outflow needle was facilitated by a roller pump (LKB 12000 Vario Perpex Peristaltic Pump) at the same rate as the inflow. The outflow was collected every 5 min in a fraction collector (Type 3403 B, RadiRac, LKB Instrument Ltd.). During the entire experiment, blood gases were measured at 15 min intervals in a BMS Mk2 Blood Micro System with a PHM 72 Mk2 Digital Acid-Base Analyzer (Radiometer, Copenhagen). Systemic blood pressure was recorded continuously via the polyethylene catheter placed in the aorta. The CSF infusion pressure and blood pressure were displayed on a Grass Model 7 Polygraph.

The perfusion fluid contained ^{14}C -inulin (inulin- ^{14}C)-carboxylic acid, $1 \mu\text{Ci}/100$

ml; Radiochemical Centre, Amersham). The ventricular system was perfused until a steady state was reached. This was shown by a plateau in the ^{14}C -activity of the effluent from the perfusion and required about 2 hrs. Samples were then collected over a further period of up to 2 1/2 hrs. Owing to the time lag in the response, also a function of the ventriculocisternal perfusion technique, the new plateau was established gradually, and all data were therefore taken at steady state, i.e. during the last hour. Inflow and effluent samples (150 $\mu\text{l}/5$ min) was mixed with 6 ml of scintillation solvent (Insta-Gel; Packard Instrument Co.), and radioactivity was measured by liquid scintillation spectrometry (Nuclear-Chicago) with quench corrections according to the external standard channels ratio method.

Treatment of data. Dilution of the ^{14}C -inulin concentration within the perfused ventricular system was used to calculate the rate at which nascent (inulin-free) CSF was added to the ventricles, according to Heisey, Held and Pappenheimer (13):

$$V_f = V_i \times \frac{c_i - c_o}{c_o} \mu\text{l}/\text{min},$$

where V = rate of flow; i , o = subscripts referring to inflow and outflow; f = subscript referring to formation of fluid, and c = concentration of radioactivity.

The protocol for each experiment consisted of one control perfusion period (during which normal CSF production rate was usually determined) and one period following drug-induced interference with the production. In some experiments a further perfusion period was included to test the effect of a second compound. The individual absolute values for the altered production rates were evaluated statistically in comparison with the control rate for each animal using Student's t-test based on paired observations, and were expressed as per cent increase or reduction from the control values. Differences between groups were compared on the basis of values from the per cent changes using the conventional Student's t-test.

Drugs. Acetazolamide (Diamox, Lederle), L-arterenol hydrochloride (Sigma), H80/62 (Prenalterol, Hässle), terbutaline sulfate (Bricanyl, Draco), phentolamine methanesulfone (Regitin, Hässle-Ciba-Geigy), propranolol chloride (Inderal, Scanmeda), practolol (Eraldine, I.C.I.).

The drugs were given either in the perfusion fluid or i.v. For the latter route they were dissolved in a Krebs-Ringer phosphate buffer which contained 30 mg % ascorbic acid to minimize oxidation. The solutions were administered either as single injections or by continuous infusion at rates given below. For the intraventricular route they were dissolved in the radioactive CSF buffer (which also contained 30 mg % ascorbic acid).

Methodological control experiments. The methodological error and any inadvertent alteration in the CSF production rate occurring during the individual experiments were checked in studies where the rate of CSF production was determined twice in the same animal by infusion of radioactive CSF buffer during two subsequent periods mimicking the control period and the subsequent experimental period following drug-induced alteration of the CSF production.

In order to evaluate the intraventricular distribution of the infused artificial CSF, experiments were performed with 50 mg % of methylene blue or trypan blue added to the CSF buffer. The experiments were either performed separately (perfusion time up to 1 hr) or they finished the perfusion experiments with radioactive buffer. After sacrificing the animals, the skull was opened and the various ventricular and subarachnoid compartments inspected for macroscopic staining.

RESULTS

The distribution of stained material (trypan blue or methylene blue) in the infusion system showed that all four ventricles were perfused from the cannula in the right lateral ventricle, the least efficient perfusion occurring in the contralateral ventricle, and that only little entered the subarachnoid space via the cisterna magna owing to the efficient drainage. The normal CSF production was determined before administration of the various drugs. In some experiments with amine receptor antagonists the compound was present already in the initial perfusion solution (after it had been established that the antagonist in question did not in itself affect the CSF production rate). The drug effect in the individual experiments is expressed as the percent change from control flow (obtained during the first perfusion period). Calculation of the mean control flow from the various experiments ($n = 122$) gave a value of 11.3 ± 0.3 (SEM) $\mu\text{l}/\text{min}$. The prolonged perfusion of artificial CSF (representing the experimental period following the control period), including changes of syringes (to mimic the situation when intraventricular administration of the test drug was started), resulted in a reduction in the CSF formation rate by $8 \% \pm 3 \%$ (mean $\pm$ SEM; $n = 7$). I.v. injection of the carbonic anhydrase inhibitor, acetazolamide (100 mg/kg), under steady state conditions produced a mean reduction in the CSF formation of 46 %.

Increasing concentrations of norepinephrine was given in the ventriculocisternal perfusion system. The physiological parameters determined are summarized in Table I. A fall in the CSF production rate was found at all amine concentrations (Fig. 1). The log dose-response curve seemed to comprise two phases with a transition above 10^{-7} M. As shown in Fig. 2, both the α -receptor antagonist, phentolamine (10^{-6} M), and the β -receptor antagonist, propranolol (10^{-6} M), counteracted the reduction in the CSF production obtained after intraventricular infusion of norepinephrine (10^{-7} and 10^{-6} M); in the presence of the latter antagonist, production was even increased.

Norepinephrine produced a reduction in the rate of CSF formation also when infused i.v. (Fig. 3). An increase in systemic blood pressure (MABP) was always seen at the beginning of the infusion (40 - 100 mm Hg), but the pressure normalized within 5 - 15 min. Other physiological parameters are summarized in Table II. Phentolamine, whether given intraventricularly (10^{-6} M) or i.v. (1 mg/kg), markedly counteracted the effect of i.v. norepinephrine (given at a rate of 14 or 27.5 μ g/kg/min). This is illustrated in Fig. 4. Phentolamine had itself little or no systemic effect, but it blocked the blood pressure increase induced by norepinephrine. As also seen in Fig. 4, propranolol counteracted the norepinephrine effect when given in the perfusion system (10^{-6} M), and markedly enhanced the norepinephrine-induced (14 μ g/kg/min) reduction in the CSF formation when given i.v. (1 mg/kg). The systemic effects were reflected in a transient slight potentiation of the blood pressure increase caused by norepinephrine.

Intraventricular administration of compound H80/62, which primarily activates the β_1 -type of receptors, reduced the CSF production rate in a dose-dependent manner (Fig. 5). When given i.v. (14 μ g/kg/min), on the other hand, it had little or no effect on the production rate. The response to H80/62 (10^{-6} M) was blocked by 10^{-4} M practolol (Fig. 2). A β -receptor agonist which mainly stimulates the β_2 -type of receptors was also tested: doses of 10^{-6} of 10^{-4} M terbutaline infused intraventricularly had no significant effect on the CSF formation; when given systemically (14 μ g/kg/min) it tended to reduce the formation, though the effect was not statistically significant.

The infusion pressure was constant, or showed a progressive increase (cf. Ref. 10), so that the mean value during the last half hour of the control period was approximately 25 mm H₂O. Under the conditions of drug-induced reduction in CSF production rate there was a tendency to a slight continuous fall (by about 10 mm H₂O, sometimes up to 30 mm H₂O) in the pressure.

DISCUSSION

The ventriculocisternal perfusion technique developed by Pappenheimer and co-workers (13, 23), based on the dilution of a non-diffusible radioactive tracer, is the most widely used and reliable method for quantitative determination of the bulk formation of CSF in the ventricular system (cf. Ref. 25). Radioactive inulin is conveniently applied as the inert tracer which fulfils required criteria for negligible degree of escape from the CSF system during its passage from one of the lateral ventricles to cisterna magna (4, 20). Since almost all inulin is lost from CSF by bulk absorption distal to the fourth ventricle, it follows that net formation of CSF is equal to the inulin clearance plus the difference between outflow and inflow rates (13). The validity of the technique and various methodological aspects of the procedure have been elucidated in detail (4, 6, 13, 23, 25). It was originally devised for use in unanesthetized goats, but has in the present study successfully been adopted for anesthetized rabbits with minor modifications (1). Since our study does not primarily aim at a determination of absolute values for the production, but rather compares drug-induced changes in the rate of CSF formation with individual control levels, any small escape of tracer from the perfusion system can be neglected as long as it remains constant.

The cerebral blood flow, and probably also the flow in the choroid plexus, is highly sensitive to alterations in blood gas tensions (14), and it has been indicated that the CSF production may vary with marked changes in PaCO_2 (18, 21). Metabolic alkalosis and hypoxia induce a decrease in the rate of CSF formation (15, 19, 21). Care was therefore taken in the present study to maintain pH, PaCO_2 and PaO_2 at a constant normal level by frequent measurements of these parameters and adjustment of respiratory rate if required. Even though it is not unlikely that also the plexus blood flow is kept constant within wide variations in systemic arterial blood pressure due to the cerebrovascular autoregulatory mechanisms, aortic blood pressure was monitored continuously to avoid too wide drug-induced fluctuations in pressure, which could have a direct influence on plexus blood flow, or might be associated with unwanted reflex activation processes (2) thereby masking local pharmacological action within the choroid plexus. Also the infusion pressure of the artificial CSF was continuously recorded as a technical control and in order to visualize any alterations in intraventricular pressure — as a result of the altered CSF production rate, under conditions when inflow and outflow in the perfusion system are constant — during the pharmacological interference with the CSF production rate. There is reason to believe that increased intracranial pressure

in itself does not significantly affect CSF formation (5).

There is evidence to indicate that changes in body temperature affects CSF production rate (7, 12, 26, 28), and it has been reported that a change of 1°C in rectal temperature (of cats) alters the rate of production by as much as 11 % (26). Body temperature was therefore carefully controlled during the entire perfusion experiment.

The mean production rate of CSF during control conditions ($11.3\ \mu\text{l}/\text{min}$) agrees well with figures reported by others (4, 6, 25). The prolonged perfusion of artificial CSF (representing the experimental period following the control period), resulted in a reduction in the CSF formation rate by 8 % (cf. refs. 18 and 19). However, in the actual calculations of drug-induced changes in CSF production, the change in each experiment was directly related to the values from the control period, thus including this error. Administration of acetazolamide, in a dose which has been found to produce a very marked reduction in carbonic anhydrase activity of the rabbit choroid plexus (8), resulted in the expected decrease in the CSF formation (4, 6, 25).

Intraventricular infusion of norepinephrine gave a dose-related reduction in the CSF production rate to a level that was 42 % lower than in the controls at an amine concentration of $10^{-3}\ \text{M}$. A similar degree of response has been reported in experiments on cats (29), and it corresponds well to the fall obtained during bilateral stimulation of the sympathetic trunks in the neck of rabbits (16). It has been shown that there is an efficient active uptake of norepinephrine into the choroid plexus (27). However, intraventricular norepinephrine was never found to induce any systemic circulatory effects, probably because the amine was, among other things, metabolized by monoamine oxidase and catechol-O-methyltransferase within the choroid plexus (17).

The antagonism of the norepinephrine response produced by either intraventricular phentolamine or propranolol in the same direction suggested that both α - and β -adrenoceptors were involved. In fact, the dose-response curve seemed to consist of two components with a transition above $10^{-7}\ \text{M}$ of infused norepinephrine. It is suggested that the fall in CSF formation at the lower norepinephrine concentrations primarily represents a β -receptor mediated effect on the secretory epithelium, whereas at higher concentrations also an α -receptor mediated local vasoconstriction (9) assists in the reduced CSF production by decreasing the plexus blood flow (4, 29). Accordingly, i.v. infusion of norepinephrine also reduced CSF production along a simple dose-response curve. Again, this effect was counteracted by phentolamine. Blockade of the β -receptors by i.v. propranolol, however, potentiated (whereas intraventricular propranolol antagonized)

the norepinephrine-induced reduction. Comparison of the intraventricular effects of the β -receptor agonists, H80/62 (3) and terbutaline (24), suggested that the β -receptor mediated reduction in CSF formation—presumably obtained by a direct inhibitory action on the plexus epithelium—mainly involved the β_1 -type of receptors.

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NE conc. (M)	Before NE infusion				During NE infusion			
	MABP (mm Hg)	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)	MABP (mm Hg)	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)
10 ⁻³	105 ± 1 (8)	7.52 ± 0.01 (6)	25.0 ± 0.6 (6)	99 ± 5 (6)	111 ± 3 (8)	7.44 ± 0.03 (7)	29.4 ± 2.0 (7)	114 ± 7 (7)
10 ⁻⁴	110 ± 7 (20)	7.47 ± 0.01 (15)	30.3 ± 0.6 (15)	119 ± 4 (15)	112 ± 8 (20)	7.41 ± 0.02 (24)	30.9 ± 0.3 (24)	117 ± 2 (24)
10 ⁻⁵	124 ± 5 (12)	7.43 ± 0.02 (28)	31.0 ± 0.9 (13)	108 ± 3 (14)	126 ± 6 (10)	7.42 ± 0.02 (15)	30.6 ± 1.2 (20)	112 ± 3 (20)
10 ⁻⁶	124 ± 3 (12)	7.51 ± 0.01 (17)	31.5 ± 0.8 (19)	112 ± 3 (19)	130 ± 2 (12)	7.43 ± 0.01 (15)	31.9 ± 0.6 (21)	111 ± 3 (21)
10 ⁻⁷	141 ± 4 (12)	7.43 ± 0.02 (22)	31.9 ± 0.9 (21)	114 ± 3 (16)	144 ± 5 (10)	7.40 ± 0.02 (14)	30.7 ± 0.8 (14)	115 ± 5 (9)
10 ⁻⁸	132 ± 4 (7)	7.43 ± 0.01 (15)	32.5 ± 1.1 (15)	127 ± 4 (15)	131 ± 5 (7)	7.40 ± 0.02 (18)	30.6 ± 0.8 (19)	141 ± 7 (19)
10 ⁻⁹	128 ± 4 (10)	7.33 ± 0.01 (10)	34.8 ± 1.9 (10)	134 ± 8 (10)	127 ± 6 (10)	7.36 ± 0.01 (12)	31.7 ± 1.2 (13)	123 ± 7 (13)

TABLE 1. Physiological variables (mean ± S.E.M. of values obtained at 15 min. intervals; number of determinations within parenthesis) during the ventriculocisternal perfusion periods, before and during the presence of various concentrations of norepinephrine (NE) to the infusate. See further Fig. 1.

NE conc. ($\mu\text{g/kg/min}$)	Before NE infusion				During NE infusion			
	MABP (mm Hg)	pH	PaCO_2 (mm Hg)	PaO_2 (mm Hg)	MABP (mm Hg)	pH	PaCO_2 (mm Hg)	PaO_2 (mm Hg)
5.5	112 $\pm$ 5 (11)	7.46 $\pm$ 0.02 (12)	34.4 $\pm$ 1.6 (12)	137 $\pm$ 4 (12)	127 $\pm$ 3 (11)	7.39 $\pm$ 0.03 (9)	34.1 $\pm$ 1.1 (14)	127 $\pm$ 4 (14)
14.0	132 $\pm$ 3 (16)	7.45 $\pm$ 0.02 (12)	30.2 $\pm$ 0.9 (19)	122 $\pm$ 5 (22)	143 $\pm$ 5 (16)	7.27 $\pm$ 0.01 (17)	32.0 $\pm$ 0.7 (29)	106 $\pm$ 2 (28)
27.5	136 $\pm$ 4 (15)	7.36 $\pm$ 0.04 (2)	33.5 $\pm$ 1.4 (18)	116 $\pm$ 2 (17)	137 $\pm$ 3 (14)	7.12 $\pm$ 0.12 (2)	32.8 $\pm$ 1.2 (17)	116 $\pm$ 2 (18)
55.0	95 $\pm$ 7 (6)	7.51 $\pm$ 0.03 (4)	33.4 $\pm$ 1.1 (6)	138 $\pm$ 9 (6)	101 $\pm$ 5 (6)	7.18 $\pm$ 0.18 (2)	32.3 $\pm$ 3.1 (5)	150 $\pm$ 4 (7)

TABLE II. Physiological variables (mean $\pm$ S.E.M. of values obtained at 15 min. intervals; number of determinations within parenthesis) during the ventriculocisternal perfusion periods, before and during i.v. infusion of various concentrations of norepinephrine (NE). See further Fig. 3.

Legends to illustrations

Fig. 1. Changes in the rate of CSF production during intraventricular perfusion of norepinephrine at various concentrations in the mock CSF infusate. The values are related to the control level of CSF production obtained in each animal prior to the amine administration (without compensating for any methodological errors, amounting to a mean of 8 %, related to e.g. change of syringe and/or the long total infusion period). Values are mean per cent levels $\pm$ S. E. M., number of animals within parenthesis. Significant differences from the control level according to the paired t-test (based on the absolute values for the CSF formation): $**0.001 < p < 0.01$. The various physiological parameters determined are summarized in Table I.

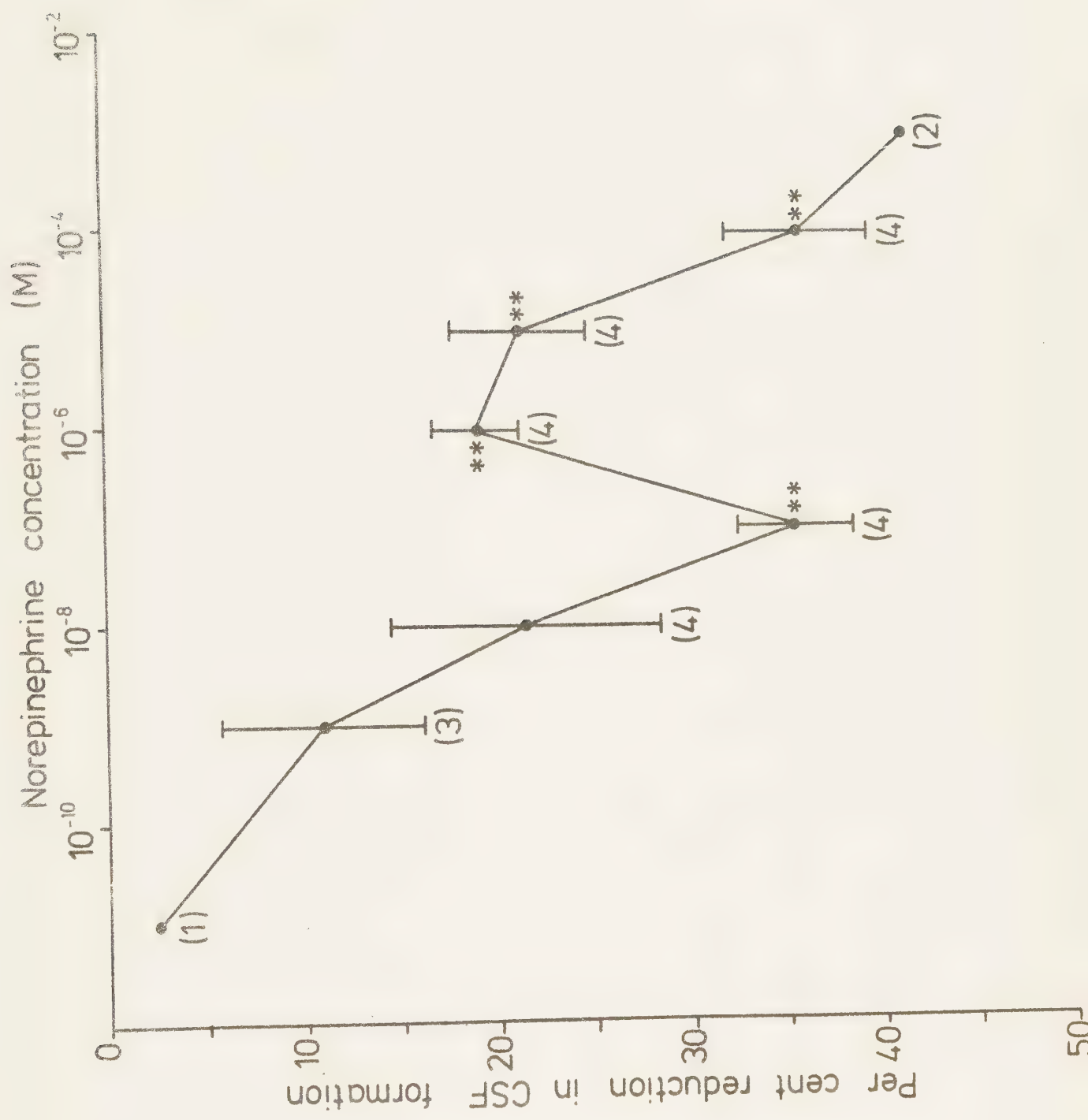
Fig. 2. Effect of various adrenoceptor antagonists (open bars) on the reduced CSF formation induced at different levels of norepinephrine (NE) or H80/62 (primarily stimulating β_1 -receptors) as agonists (hatched bars). Concentrations given are the molar concentrations in the infusate. Values are mean per cent levels $\pm$ S. E. M., number of animals within parenthesis. Significant differences from the control level according to the paired t-test (based on the absolute values for the CSF formation): $** 0.001 < p < 0.01$, $*** p < 0.001$; differences between mean per cent agonist and antagonist values according to Student's t-test using unpaired observations: $^{oo} 0.001 < p < 0.01$, $^{ooo} p < 0.001$.

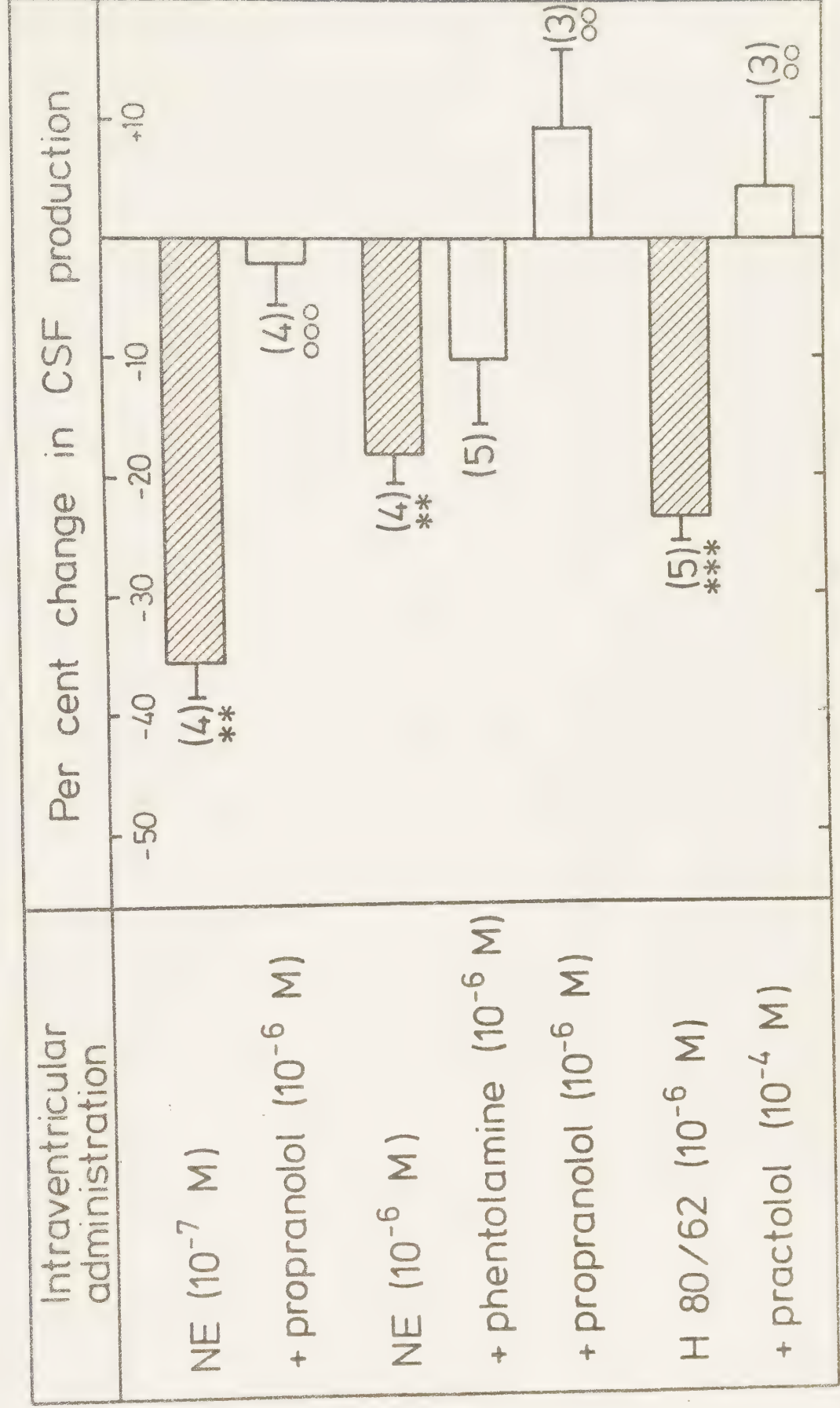
Fig. 3. Changes in the rate of CSF production during i.v. infusion of various norepinephrine concentrations. The values are obtained as in Fig. 1., and show mean per cent levels $\pm$ S. E. M., number of animals within parenthesis. Significant differences from the control level before the amine infusion according to the paired t-test (based on the absolute values for the CSF formation): $* 0.01 < p < 0.05$, $** 0.001 < p < 0.01$. The various physiological parameters are summarized in Table II.

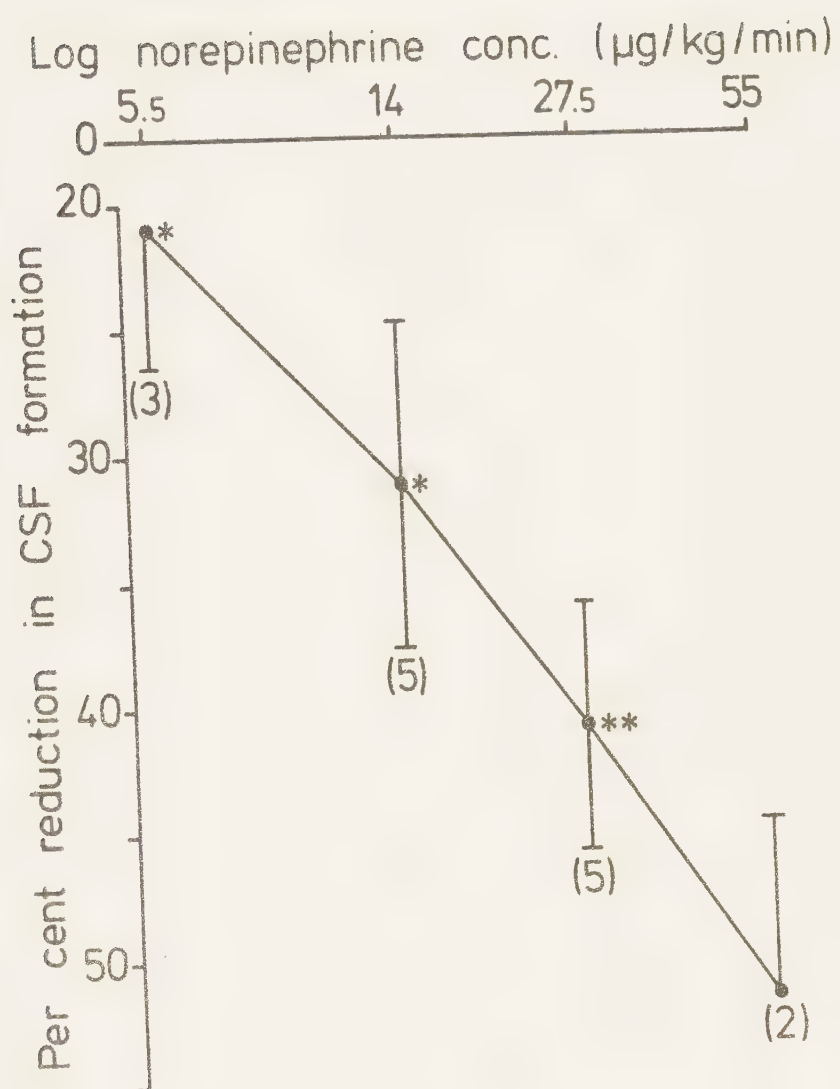
Fig. 4. Effect of various adrenoceptor antagonists (open bars) on the reduced CSF formation induced at different levels of infused norepinephrine (hatched bars). The antagonists were administered either as i.v. injections repeated at the dose indicated every 30 - 45 min during the ventriculocisternal perfusion period, or as a

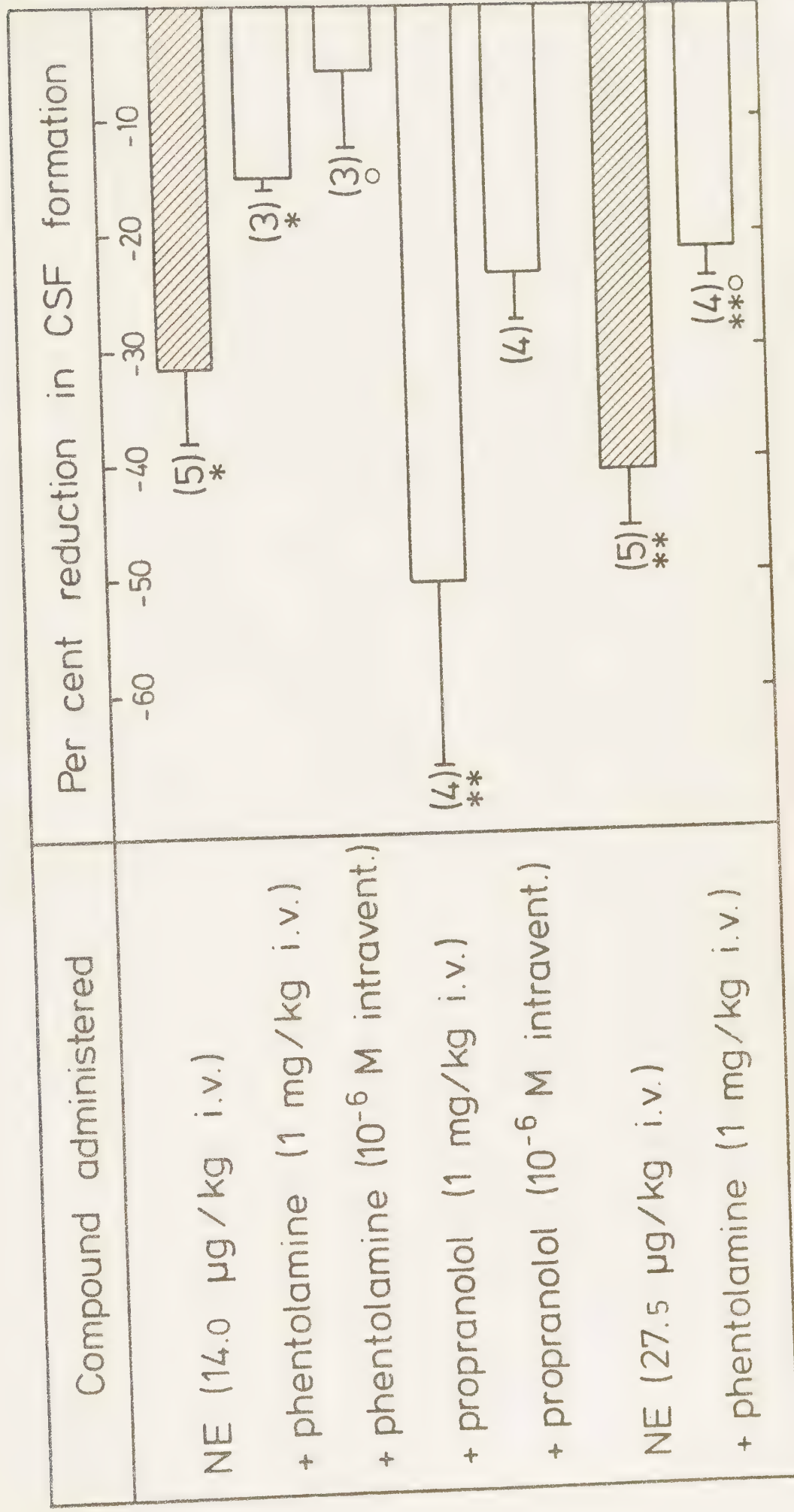
continuous intraventricular perfusion (concentration in the infused mock CSF is shown). Values are mean per cent levels + S.E.M., number of animals within parenthesis. Significant differences from the control level according to the paired t -test (based on the absolute values for the CSF formation): * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$; differences between mean per cent agonist and antagonist values according to Student's t -test using unpaired observations: $^{\circ}$ $0.01 < p < 0.05$.

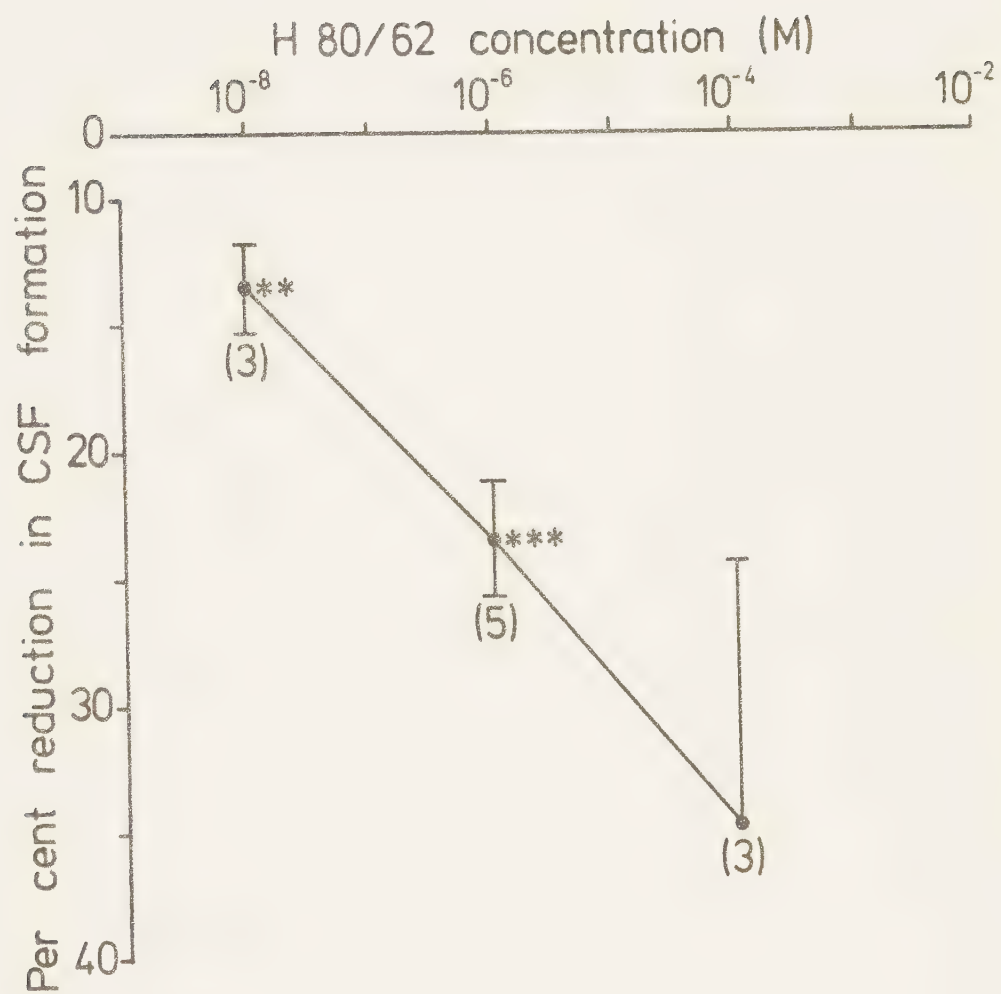
Fig. 5. Changes in the rate of CSF production during intraventricular perfusion of the β_1 -receptor agonist, H80/62, at various concentrations in the infusate. For further information, see Fig. 1. Paired t -test: ** $0.001 < p < 0.01$, *** $p < 0.001$.











EVIDENCE FOR THE PRESENCE OF TWO TYPES OF MONOAMINE OXIDASE IN RABBIT
CHOROID PLEXUS AND THEIR ROLE IN BREAKDOWN OF AMINES IN-
FLUENCING CEREBROSPINAL FLUID FORMATION

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Running title: MAO in the rabbit choroid plexus

Abstract. The presence of MAO in the choroid plexuses from all brain ventricles of rabbit was established radiometrically. Histochemical staining of MAO revealed the enzyme to be present primarily in the secretory epithelium. The mode of inhibition by the selective inhibitors, clorgyline and deprenyl, showed that type B MAO predominated in the plexus. Intraventricular administration of nialamide markedly reduced CSF production measured by ^{14}C -inulin dilution during ventriculocisternal perfusion. The nialamide effect was significantly diminished after sympathetic denervation of the plexus, indicating that also neuronal MAO (i.e. type A) is present in the plexus. In addition, nialamide tended to potentiate the reduction in CSF formation obtained by intraventricular noradrenaline.

THE secretory epithelium and blood vessels of the choroid plexus are richly supplied with sympathetic adrenergic nerves originating in the superior cervical ganglia (EDVINSSON, NIELSEN, OWMAN and WEST, 1974). Denervation experiments and electrical stimulation have shown that the sympathetic nerves in the plexus exert a marked inhibitory influence on the bulk production of CSF (LINDVALL, EDVINSSON and OWMAN, 1978a) as measured in rabbits by dilution of ^{14}C -inulin during ventriculocisternal perfusion according to HEISEY, HELD and PAPPENHEIMER (1962) and PAPPENHEIMER, HEISEY, JORDAN and DOWNER (1962). When sympathomimetic agonists were administered into the ventricles in order to establish the type of adrenoceptors involved in the response (LINDVALL, EDVINSSON and OWMAN, 1978b) it was found that the effect of noradrenaline on the CSF formation was enhanced following local inhibition of monoamine oxygen oxidoreductase (MAO; E.C. 1.4.3.4) via the perfusate. The present study shows that MAO can be demonstrated histochemically and biochemically in the rabbit choroid plexus, and that the enzyme probably assists in the regulation of an inhibitory sympathetic tone on the CSF formation.

MATERIALS AND METHODS

Animals. The material comprised 53 albinic and randomly pigmented rabbits of either sex, weighing 2.5 - 3.5 kg, maintained on standard pellet food and tap water ad lib. Sympathetic denervation of the choroid plexuses was carried out in 7 animals under nembutal anesthesia by removal of the superior cervical ganglia on both sides.

Determination of CSF production. The rate of bulk CSF production was measured in 19 animals by intraventricular dilution of ^{14}C -inulin (inulin [^{14}C]-carboxylic acid, 1 $\mu\text{Ci}/100\text{ ml}$, Radiochemical Centre, Amersham) during ventriculocisternal perfusion (HEISEY et al., 1962; PAPPENHEIMER et al., 1962). The animals were sedated with nembutal, tracheotomized, and then anesthetized with halothane and a mixture of N_2O and O_2 in an open system during artificial ventilation; the anesthesia was continued without halothane. They were placed in a prone position with the head fixed in a metal frame. Artificial CSF containing the labelled inulin was infused at a constant rate of 30 $\mu\text{l}/\text{min}$ through an inflow probe into the right lateral ventricle during continuous recording of infusion pressure by a Statham model P23 AC transducer. The fluid was drained at the same rate by means of a needle placed in the cisterna magna, and the outflow (150 μl) was collected at 5-minute intervals. Blood gases were frequently measured throughout the experiment in an Astrup system (Radiometer, Copenhagen), and systemic blood pressure was monitored continuously. Attempts were made to maintain blood gases

constant within narrow limits throughout the experiments by small adjustments in the respiratory rate. For further methodological details, see LINDVALL *et al.* (1978b). Radioactivity was measured by liquid scintillation counting, quench corrections being obtained according to the conventional principles.

Determination of MAO activity. Two series of animals were used. In one group (7 animals) the ventricular system was perfused with mock CSF without ^{14}C -inulin during a time period corresponding to that in the CSF production studies (LINDVALL *et al.*, 1978b). The perfusion medium of 4 animals contained 10^{-3} M of the MAO inhibitor, nialamide (Pfizer), and 3 animals served as controls. Samples of choroid plexus, weighing 5 – 20 mg depending on region (see Table 1), were dissected out.

No ventriculocisternal perfusion was performed in the second group consisting of 22 animals. During nembutal anesthesia, the head was perfused through injection of 500 ml saline into the ascending aorta in order to rinse the vascular bed of the choroid plexuses. The blood-free plexus tissue was dissected out from each ventricle separately (tissue weight 1 – 10 mg). Enzyme activity was determined: (a) On tissue preincubated for 20 min at 37°C in a glucose-containing (6 mM) Krebs-Ringer buffer solution with either of the selective MAO inhibitors, clorgyline (May and Baker; 10^{-5} M) and deprenyl (Ferrosan; 10^{-5} M); some of the deprenyl-treated tissue had been subjected to sympathetic denervation 12 days beforehand. (b) On control tissue preincubated in the buffer solution alone (see Table 2). The tissues were washed in ice-cold buffer solution, and gently blotted on filter paper.

Plexus tissue obtained from either of the two groups was frozen in liquid nitrogen to facilitate cell disruption. After thawing, the tissue was homogenized in 10 mM sodium phosphate buffer (pH 7.0) for biochemical assay of MAO activity (FONNUM, 1976). Tissue protein was determined according to LOWRY, ROSEBROUGH, FARR and RANDALL (1951). The assay was linear with respect to protein concentration and incubation time.

Histochemistry of MAO activity. MAO was demonstrated histochemically in 5 animals using 20 μ thick cryostat sections (GLENNER, BURTNER and BROWN, 1957). Controls were obtained by omitting the substrate (tryptamine) from the incubation solution or by pretreatment of 2 of the animals with 100 mg/kg i.p. of the MAO inhibitor, nialamide, 1 hr before sacrifice.

RESULTS AND COMMENTS

The choroid plexus had biochemically measurable activity of MAO (Table 1). Ventriculocisternal perfusion of the MAO inhibitor, nialamide, at the concentration of

10^{-3} M for 1.5 - 2 hrs reduced the enzyme activity in the plexus tissue by 87 %. The amount of inhibition was of the same magnitude in the plexus from that ventricle containing the cannula compared to the other plexuses (Table 1).

Histochemical staining of sections from plexus tissue showed the presence of tetrazolium precipitate, representing enzyme activity at the cellular level, in high concentration within the cytoplasm of the plexus epithelium of all ventricles. Little or no staining was seen in the walls of larger vessels. The control sections were negative. This shows that the bulk of the MAO activity in the choroid plexus is present in the epithelial cells rather than in the vascular smooth musculature or the adrenergic nerves (cf. ALMGREN, ANDÉN, JONASON, NORBERG and OLSON, 1966).

Administration of 10^{-4} M noradrenaline (L-arterenol hydrochloride; Sigma) in the ventriculocisternal perfusion system during 2 hrs (the time chosen to obtain steady state conditions in the ^{14}C -inulin dilution) reduced the CSF production rate by 36 % (Fig. 1). This represents a near maximum response in the dose-related fall in the production (LINDVALL et al., 1978b). Pretreatment with 10^{-3} M nialamide in the perfusion system, which by itself reduced the CSF formation by 44 % compared with the control period without nialamide, potentiated the effect of noradrenaline, now resulting in a 65 % lower production rate than during control conditions (Fig. 1).

The unexpectedly pronounced effect of nialamide alone would indicate that, besides any unspecific action nialamide may have on the CSF formation, the normal rate of production is under the influence of a substantial "sympathetic tone". In order to test the importance of the sympathetic nerve activity, the effect of nialamide perfusion on the CSF production was tested after sympathetic denervation of the choroid plexuses by removal of the superior cervical ganglia. Under these conditions nialamide only reduced the production by 22 % (3 animals) compared to the control situation in the non-sympathectomized animals. It has previously been determined that sympathectomy alone increases the production by 33 % (LINDVALL et al., 1978a).

In order to distinguish the type of MAO present in the choroid plexus, tissues were preincubated together with selective inhibitors (NEFF and YANG, 1974). Clorgyline, which primarily inhibits the A type of the enzyme, had only a slight effect on the activity, whereas the type B inhibitor, deprenyl, markedly reduced the enzyme activity compared with control tissue (Table 2). The MAO activity remaining in sympathectomized plexuses treated with deprenyl was similar to the activity found after deprenyl preincubation of intact plexuses (Table 2).

DISCUSSION

Since diffusion of inulin from the ventricular system is negligible, it follows that any dilution of the radioactive tracer during passage through the ventricles results from newly formed, inulin-free fluid at a rate equal to the rate of inflow times the difference between the inulin concentrations in the inflow and outflow, divided by the concentration in the outflow (HEISEY *et al.*, 1962). The mean bulk production of CSF in the untreated rabbit was found to be 10.8 – 11.3 $\mu\text{l}/\text{min}$ (LINDVALL *et al.*, 1978a and b), which is in good agreement with previously published figures (for reference, see DAVSON, 1967; CSERR, 1971).

Ventriculocisternal perfusion of noradrenaline reduced the rate of CSF production. The dose of the amine was chosen to give a near maximum response, providing an inhibition which probably involves both a direct effect on the secretory epithelium and a reduced blood perfusion of the tissue (LINDVALL *et al.*, 1978b). Infusion of the (non-selective) MAO inhibitor, nialamide, also markedly inhibited the CSF formation, probably by an effect on the MAO found by biochemical and histochemical methods to be present in the choroid plexus. Pretreatment with nialamide tended to potentiate the noradrenaline-induced reduction of the CSF formation. It should be noted though, that the degree of reduction in the CSF formation in a prolonged perfusion experiment, consisting of a control period followed by one period of nialamide administration and one during which the inhibitor is given together with noradrenaline, is slightly overestimated. Perfusion during 5 – 6 hrs corresponding to the above situation, was found to reduce the CSF production by a mean of 16 % (3 animals) also in the absence of drugs. The time-dependent fall measured from the last half hour in the control period to the last half hour in the subsequent experimental period represented 13 %. The remaining 4 % of the fall in CSF formation occurred during the third period of the entire perfusion experiment.

Two types of MAO have been distinguished with different substrate preference and different sensitivity to the selective inhibitors, clorgyline and deprenyl (NEFF and YANG, 1974; EGASHIRA, EKSTEDT and ORELAND, 1976). Sympathetic nerves contain primarily type A MAO (GORIDIS and NEFF, 1971a and b), whereas the B type of the enzyme has mainly an extraneuronal localization (cf. LEVIN and WILSON, 1977). The present finding that the type B selective inhibitor, deprenyl, markedly reduced plexus MAO activity — to a similar extent also after sympathetic denervation — and that clorgyline (which selectively affects MAO A) had a comparatively much less pronounced action,

suggests that the majority of plexus MAO is of the B type. However, the finding that sympathectomy reduced the nialamide effect on the CSF formation indicates that sympathetic nerve MAO (*i.e.* type A) is also present. In addition to MAO, the rabbit choroid plexus also shows biochemically measurable activities of the enzyme S-adenosyl-L-methionine catechol O-methyltransferase (catechol O-methyltransferase; E.C. 2.1.1.6) (unpublished observations).

The effect of sympathetic denervation on the nialamide-induced reduction of the CSF formation indicates that this is under the influence of a substantial inhibitory sympathetic tone under the prevailing steady-state conditions. Some of the MAO activity in the choroid plexus may be involved also in the breakdown of intraventricular noradrenaline (ZIEGLER, LAKE, FOPPEN and SHOULSON, 1976), which is actively taken up by the plexus, probably into its epithelium (TOCHINO and SCHANKER, 1965). However, noradrenaline is not a preferred substrate for MAO B (NEFF and YANG, 1974). The effect of nialamide that was found in the sympathectomized animals may hence also be the result of — besides any unspecific inhibition of the secretion by the drug itself — an impaired breakdown by MAO (or some other nialamide-sensitive degrading enzyme) of other compounds, locally formed in the plexus epithelium or taken up from the CSF, having an inhibitory effect on its formation. Similar functions may be exerted by the catechol O-methyltransferase in the choroid plexus. It is even possible that both enzymes, together with the tight junctions between the plexus epithelial cells (see BRIGHTMAN, 1975), are involved in a gate mechanism for circulating amines that have passed beyond the fenestrated endothelial lining of the plexus.

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TABLE 1. MAO ACTIVITY IN THE CHOROID PLEXUS FROM UNTREATED RABBITS
AND AFTER VENTRICULOCISTERNAL PERFUSION WITH NIALAMIDE

Treatment	Ventricle	I + III + IV (cpm/ μ g protein)*	II (cpm/ μ g protein)	I + II + III + IV (cpm/ μ g protein)
Untreated		-	-	311 $\pm$ 64 (3)
Nialamide (10^{-3} M)**		45 $\pm$ 6 (3)	50 $\pm$ 15 (3)	39 $\pm$ 9 (4)

* Results are expressed as mean $\pm$ S.E.M., number of animals within parenthesis.

** The cannula used for the perfusion was placed in the right lateral ventricle (II), outflow was provided through a needle in the cisterna magna.

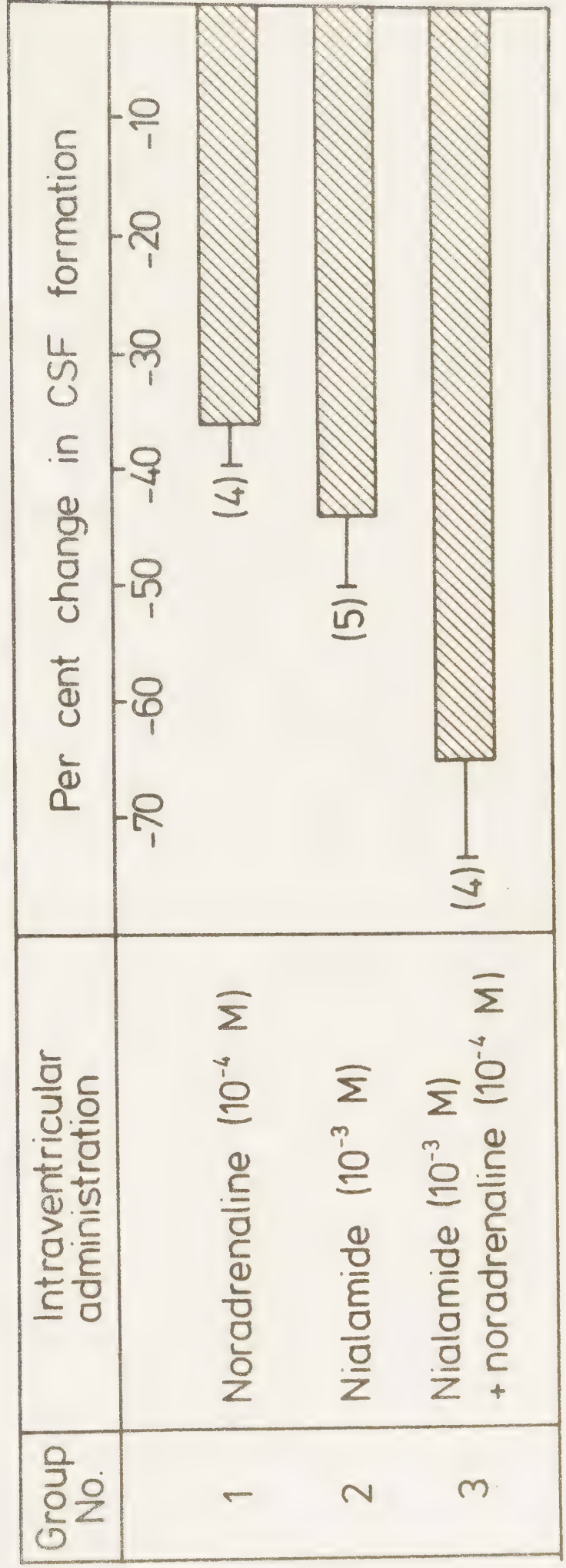
TABLE 2. TWO SERIES OF EXPERIMENTS SHOWING PER CENT REMAINING ACTIVITY OF PLEXUS MAO DETERMINED AS cpm/ μ g PROTEIN: (a) FOLLOWING PREINCUBATION IN THE PRESENCE OF THE MAO INHIBITORS, CLORGYLINE (INHIBITS PRIMARILY TYPE A) AND DEPRENYL (INHIBITS PRIMARILY TYPE B) COMPARED WITH CONTROLS PREINCUBATED IN BUFFER SOLUTION ALONE, AND (b) IN DEPRENYL-INCUBATED INTACT AND SYMPATHECTOMIZED PLEXUSES COMPARED WITH CONTROLS.

Inhibitor Region (ventricle)	Clorgyline 10 ⁻⁵ M	Deprenyl 10 ⁻⁵ M	Deprenyl 10 ⁻⁵ M (intact plexus)	Deprenyl 10 ⁻⁵ M (sympathectomized plexus)
Lateral (I + II)	70 %	19 %***	19 %***	18 %***
III	100 %	26 %*	27 %**	21 %***
IV	85 %	35 %**	34 %*	42 %*
All ventricles	80 %	24 %***	23 %***	22 %***

Statistical evaluation of the effect of MAO inhibitors compared with controls according to Student's *t*-test:

p* < 0.05; **0.001 < *p* < 0.01; **p* < 0.001.

Fig. 1 Amount of reduction in the bulk CSF formation during various experimental conditions. The molar concentrations in the perfusate are given. Values are mean per cent levels + S.E.M., number of animals within parenthesis. Differences from the control level according to the paired t-test (based on the absolute values for the CSF formation): $0.001 < p < 0.01$ in all groups. Comparison (mean per cent values) of levels in groups 1 and 2 with that of group 3 (Student's t-test on unpaired observations): $p < 0.05$.



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AUTONOMIC NERVOUS CONTROL
OF
CEREBROSPINAL FLUID PRODUCTION
FROM
THE CHOROID PLEXUS

by

MARIA LINDVALL

LUND 1979

To my parents,
Marianne and Gustaf Lindvall

The present thesis is based on the following papers:

- I Fluorescence histochemical study on regional differences in the sympathetic nerve supply of the choroid plexus from various laboratory animals. Cell Tiss. Res., in press, 1979.
- II Early development of noradrenaline-containing sympathetic nerves in the choroid plexus system of the rabbit. Cell Tiss. Res. 192, 195-203, 1978 (together with Ch. Owman).
- III Histochemical study on regional differences in the cholinergic nerve supply of the choroid plexus from various laboratory animals. Exp. Neurol. 55, 152-159, 1977 (together with L. Edvinsson and Ch. Owman).
- IV Ultrastructural and biochemical evidence for a sympathetic neural influence on the choroid plexus. Exp. Neurol. 48, 241-251, 1975 (together with L. Edvinsson, R. Håkanson, Ch. Owman and K.-G. Svensson).
- V Autonomic vascular innervation and vasomotor reactivity in the choroid plexus. Exp. Neurol. 62, 394-404, 1978 (together with L. Edvinsson).
- VI Sympathetic nervous control of cerebrospinal fluid production from the choroid plexus. Science 201, 176-178, 1978 (together with L. Edvinsson and Ch. Owman).
- VII Effect of sympathomimetic drugs and corresponding receptor antagonists on the rate of cerebrospinal fluid production. Exp. Neurol., in press, 1979 (together with L. Edvinsson and Ch. Owman).

- VIII Evidence for the presence of two types of monoamine oxidase in rabbit choroid plexus and their role in breakdown of amines influencing cerebrospinal fluid formation. J. Neurochem. , under publication, 1979 (together with Ch. Owman).
- IX Reduced cerebrospinal fluid formation through cholinergic mechanisms. Neuroscience Letters 10, 311-316, 1978 (together with L. Edvinsson and Ch. Owman).

These papers will be referred to by their Roman numerals.

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1. INTRODUCTION

The choroid plexus, present in all four ventricles of the mammalian brain, plays an important and probably major role in the production of cerebrospinal fluid (CSF) (for reviews, see Davson, 1967; Dohrmann, 1970; Cserr, 1971; Milhorat, 1975). There is evidence that the ependymal lining of the ventricles and the central canal of the spinal cord (Pollay and Curl, 1967; Sonnenberg et al., 1967), and the cranial and spinal subarachnoid space (Sato and Bering, 1967; Sato et al., 1972), constitute extrachoroidal sites of CSF production and exchange with the entire interstitial space of the CNS parenchyma (see also Milhorat, 1975).

That the CSF is formed by active secretion from the choroid plexus is supported by the morphological structure of the tissue. It is villous, highly vascularized, and covered by a single layer of cuboidal cells supplied with various membrane elaborations (apical microvilli, basal and basolateral infoldings and interdigitating processes) and numerous mitochondria. In this respect it much resembles other well-known fluid transporting epithelia. The choroid epithelium is sealed by tight junctions, and the capillaries are unusually wide, thin-walled and fenestrated.

The production of CSF is an energy-requiring secretory process involving at least two enzymes, carbonic anhydrase and an ouabain-sensitive sodium-potassium activated ATPase (for review, see Cserr, 1975). The mechanisms controlling the CSF production during physiological conditions are still poorly understood, but a large number of drugs and experimental conditions have been shown to influence the normal rate of formation, mostly in the direction towards a decreased production (Pollay, 1975). Blood flow has been suggested by Pappenheimer (see Ames et al., 1965) to influence the secretory rate by imposing an upper limit, and it is possible that at least some of the factors that affect the CSF secretion do so by altering the plexus blood flow (Cserr, 1971).

The first report about nerves in the choroid plexus was published about one hundred years ago (Benedikt, 1874). Later morphological studies con-

firmed these observations, although publications on the plexus innervation are, on the whole, rather few. However, in 1928 Clark suggested that the nerve endings he could visualize near the epithelial cells were secretory, and Shapiro discussed the role of the autonomic nervous system in the secretory process already in 1931.

The brain is enclosed in a rigid compartment, and intracranial pressure dynamics therefore constitute a physiological parameter of fundamental importance for a normal brain function and of great clinical significance. The intracranial pressure is governed by the cerebral blood volume and the CSF circulation. The CSF also constitutes a mechanism of exchange between the blood and brain as well as a route of transport within the brain itself, functions whose importance has been considerably emphasized during the last few years.

The present knowledge about the cellular mechanisms underlying the formation of CSF are fairly well understood, whereas our understanding about the various control mechanisms regulating the CSF circulation is surprisingly poor (Davson, 1967; Cserr, 1971 and 1975; Pollay, 1975), particularly in view of the serious clinical conditions that become the result of an impaired flow of CSF. Thus, besides surgical drainage of CSF, pharmacological treatment of hydrocephalus has been of little success, and it has involved crude approaches such as administration of acetazolamide (for further discussion, see Plum and Siesjö, 1975).

Investigations of the possible involvement of neurogenic mechanisms in the control of CSF formation from the choroid plexus has to a large extent been hampered by the widely held opinion that the choroid plexus is a non-innervated structure. However, the use of modern neurohistochemical techniques has revealed that the plexus is indeed supplied with an extensive system of autonomic nerves (Edvinsson et al., 1973 and 1974). Against the background of these observations a series of investigations was carried out with the following aims:

- (1) to study the origin, detailed distribution and ontogenetic development of adrenergic nerves in the mammalian choroid plexus,

- (2) to compare the supply of cholinergic nerves with the system of adrenergic fibres in the plexus tissue,
- (3) to analyse the ultrastructural relationship between the autonomic nerve terminals and the two major effector components in the choroid plexus — the smooth muscle cell of the vascular walls and the secretory epithelial cell,
- (4) to study the prerequisites for and the possible importance of a functional neurogenic influence on the rate of CSF production through sympathetic stimulation and denervation,
- (5) to characterize the autonomic receptors mediating sympatho- and parasympathomimetic effects on the CSF production.

2. INNERVATION OF THE MAMMALIAN CHOROID PLEXUS

2.1. Early investigations on the presence of nerves

As far back as 1874 Benedikt identified nerve fibres both in relation to the epithelium and to the vessels in the plexus of the fourth ventricle by histological staining. These fibres were branches of the tenth nerve and had their origin in the cells of the nucleus ambiguus. He called them "the thirteenth cranial nerve". Findlay (1899) was able to demonstrate nerve fibres in the lateral choroid plexus of man and cattle, but only fibres of vasomotor nature. In 1911 Hworostuchin described a great number of nerve fibres in all four choroid plexuses of various mammals. The fibres formed networks not only on blood vessels but also just below the epithelium. From the subepithelial network, small fibres left to end on the upper surface of the epithelium. Extensive studies on the pial innervation including the choroid plexus were made by Stöhr (1922) using silver staining on human material. He divided the choroid plexus nerves into two groups — vascular nerves (nerves attached to blood vessels of all calibres) and nerves proper of the choroid plexus (not related to blood vessels). Some delicate fibres were observed below the epithelium and in close association to this, but it was impossible for him to

decide to which structure the fibres belonged. He described the vascular nerves as arising from the internal carotid and the vertebral plexuses, and from some of the cranial nerves. The nerve fibres of the choroid plexus proper had their origin in the nucleus of the 9th and 10th cranial nerve, the pontine nuclei and the cerebral peduncles. They were also described to enter from the brain substance itself into that part of the tissue which overlies the thalamus.

Junet (1926) observed not only the perivascular plexus of nerves already described by Stöhr (1922), but also subepithelial plexuses of delicate fibres and tiny fibrils which entered the epithelium and often terminated in little whirls. Further studies were made by Clark (1928) on nerve fibres and their endings in the choroid plexuses of several species and with several staining techniques. He observed fibre endings in relation to blood vessels, in the pial connective tissue, and beneath or between epithelial cells. Most nerve fibres were unmyelinated and thought to be sensory. There was a difference in the innervation of the lateral and medial portion of the plexus from the fourth ventricle. The nerves of the lateral part of the plexus had a two-fold source. Some accompanied vessels, whereas others originated in the dorsolateral part of the medulla oblongata and formed the fascicle called the thirteenth cranial nerve by Benedikt (1874). The nerve fibres of the medial portion originated in the substance of the medulla. Apart from the fibres to smooth musculature he showed the nerve fibres to follow lines of separation between epithelial cells. Similar nerve fibres and endings were also present in the choroid plexus of the lateral ventricle (Clark, 1934).

Snessarew (1929) did not agree with Stöhr's (1922) division into vascular nerve fibres and nerves proper: he suggested the former to be defined as a nerve plexus of the vascular walls and the latter as an intervascular plexus. Besides vascular fibres he found others of the terminal type, where the ramifications surrounded the epithelial cells like a network.

In the studies of Shapiro (1931) it was observed that the choroid plexus was rich in nerve fibres, but the plexus epithelium scantily supplied. It was possible to trace the penetration into the epithelium of tiny varicose nerve

fibrils, and to show their termination between the epithelial cells.

Tsuker (1947) described both "stromal nerves" scattered through the connective tissue and "vascular nerves" surrounding the vessels. The greatest number of massive nerve bundles were observed with silver staining in the connective tissue stroma of the lateral plexuses. After unilateral sympathectomy (removal of the superior cervical sympathetic ganglion) in cats and dogs, he observed degeneration of single nerve fibres in the choroid plexus of the homolateral ventricle. No degeneration was traceable in the choroid plexus of the contralateral ventricle. In the plexus of the third ventricle there was degeneration of part of the nerve fibres, too. The observations gave evidence that not all nerve fibres of the choroid plexus originate from the nerve networks of the arteries.

In conclusion the early investigators showed that the choroid plexus has an innervation where part of the nerve fibres end in relation to the vessels, part of them near the epithelial cells. Some arise from the cervical sympathetic ganglia, while others come from the glossopharyngeal and vagus nerves.

The results from the early investigations are often difficult to interpret because the histological staining methods used (mostly silver impregnation) do not visualize nerve fibres exclusively, and not until the introduction of modern neurohistochemical techniques did it become possible to define the identity, origin, and distribution of the plexus nerves more precisely.

2.2. Histochemistry of adrenergic nerves

A. Nerve supply in adult animals. The Falck-Hillarp histofluorescence technique is a highly sensitive and specific method for visualization of catecholamines in peripheral adrenergic nerves (for details, see Björklund et al., 1972). It was used in a study on regional differences in sympathetic nerve supply to the choroid plexus of nine different animal species (Paper I): pig, cat (see also Paper IV), monkey, guinea-pig, rat, rabbit (see also Papers IV and VI), cow, hamster and mouse. Edvinsson et al. (1974) showed in an

earlier study that the choroid plexus of mouse, rat, guinea-pig, rabbit, cat, cow and monkey receive adrenergic nerve terminals in close relation to both vessels and the choroid plexus epithelium. This was confirmed in Paper I, which further describes clear differences both with respect to the nerve supply of the plexus in the individual ventricles and to the total plexus supply in different species. The general pattern of distribution of the adrenergic fibres was, however, very similar in all species: the nerve terminals formed networks around small vessels and in the plexus tufts, with isolated fibres and nerve terminals running in close relation to the epithelium. The plexuses of pig and cat were most well-supplied with adrenergic nerves, monkey, guinea-pig, rat, rabbit and hamster showed an intermediary density, while cow and mouse received the fewest nerve fibres. The histochemical findings were correlated with bioenzymatic measurements of the noradrenaline (NA) content in the tissue according to Cuello et al. (1963) as modified by Coyle and Henry (1963). All examined species showing the presence of adrenergic nerve terminals in their plexuses also contained measurable amounts of NA. The transmitter content varied between 0.10 - 0.73 ng/mg tissue protein (Paper I). No quantitative determinations were made of the amine content in plexuses of different ventricles in the same species, with exception of the choroid plexus of rabbit (Paper II). In this animal the plexus of the third ventricle was found to contain 1.46 ng/mg protein, that of the lateral ventricles 0.27 ng/mg, and the plexus of the fourth ventricle 0.15 ng/mg. These values are in good agreement with the fluorescence histochemical findings, showing the innervation density to be: third ventricle plexus > lateral plexuses > fourth ventricle plexus. This order of plexus nerve density was found in all species examined (Edvinsson et al., 1974; Papers I and VI). Fluorometric determinations (Bertler et al., 1958; Häggendal, 1963) of the NA concentration related to the wet weight of plexus tissue in the rabbit gave a value of 0.4 µg NA/g tissue (Edvinsson et al., 1972; Paper VI). Bilateral sympathectomy (removal of the superior cervical ganglia) was earlier reported to abolish all plexus nerves in cat, rabbit and rat (Edvinsson et al., 1974). In Paper IV the findings

were confirmed in the cat, but in the rabbit scattered nerves remained in the fourth ventricle plexus (Papers IV and VI), probably originating from lower ganglia. The chemically measured NA level was reduced to non-significant amounts after ganglionectomy (Edvinsson et al., 1972; Paper VI). Unilateral sympathectomy showed a complete disappearance of nerves in the homolateral choroid plexus, and a marked reduction of nerves in the midline plexuses (Paper I).

B. Ontogenetic development of the nerve supply. The choroid plexus of the rabbit is well supplied with adrenergic nerves (Paper I) and was chosen for functional studies of the sympathetic influence on CSF formation (see Papers IV, VI, VII and VIII). In order to elucidate at what stage of development a sympathetic neurogenic control of CSF production might be exerted, and to provide a basis for correlation with any future physiological studies on neurogenic control of CSF formation during development, the ontogeny of adrenergic nerves in the choroid plexus from rabbit was followed (Paper II), both fluorescence histochemically with the Falck-Hillarp technique (for details, see Björklund et al., 1972) and the glyoxylic acid method (Lindvall and Björklund, 1974), and by biochemical quantification of the NA content (Cuello et al., 1963; Coyle and Henry, 1963). The choroid plexus of the rabbit has certain similarities to man in its histogenetic development (Tennyson and Pappas, 1968), but the period of structural maturation differs: in man the choroid plexus starts to mature already after the 29th week of gestation (Shuangshoti and Netsky, 1966), whereas adult characteristics in the rabbit are not seen until the early postnatal period (Tennyson and Pappas, 1964 and 1968).

No adrenergic nerves could be demonstrated prenatally with either of the two histofluorescence techniques used (Paper II). Some plexuses were incubated in the presence of α -methyl-NA (Read and Burnstock, 1969) because the possibility existed that some adrenergic nerve fibres could have escaped histochemical detection because no or too little catecholamines had yet been formed in them. The first fluorescence histochemical signs of adrenergic nerves

were found in the plexus of the third ventricle around birth, whether the tissue had been preincubated in the presence of α -methyl-NA or not. Somewhat later, around the 5th postnatal day, nerve fibres appeared also in the plexuses of the lateral ventricles, whereas the plexus of the fourth ventricle did not receive any histochemically visible nerves until around two weeks post partum (Paper II).

Nerve fibres grew along blood vessels to form a plexus of nerves located between small vessels and the epithelium. Outgrowing axons had a thick, smooth appearance with relatively high fluorescence intensity, while the developing nerve terminals initially showed very weak fluorescence intensity (Paper II). The whole choroid plexus system showed full adrenergic nerve development at three weeks post partum (Paper II) with a distribution of nerve terminals resembling that seen in adult animals (Paper I). However, the density of nerves was subsequently further increased. Correlation of biochemical measurements with the histochemical findings showed very good agreement (Table I in Paper II).

Tennyson (1975) suggested that the morphological appearance of the telencephalic choroidal epithelial cells during different stages of development reflects changes in function. Kiszely (1951) proposed on morphological basis a resorptive function of the choroid plexus during most of the embryonic life, while Flexner (1938) believed the CSF to change from an ultrafiltrate to secretion during development. The plexus epithelium of rabbit reaches mature characteristics after two weeks post partum (Tennyson and Pappas, 1968). Structural possibilities for a sympathetic neurogenic influence on CSF production thus exist already a few weeks after birth. Functional studies of the onset and maturation of neuroeffector transmission mechanisms was made by Schwieler et al. (1970), who examined heart rate and tracheal smooth muscle tone in rabbit, and by Furness et al. (1970), who studied smooth muscle function in the vas deferens of mouse. It was concluded that an autonomic efferent innervation is established early in the rabbit, and in view of the above-mentioned investigations it may be assumed that a first step in a functioning neurogenic influence on the rabbit

choroid plexus becomes established within the first three weeks after birth.

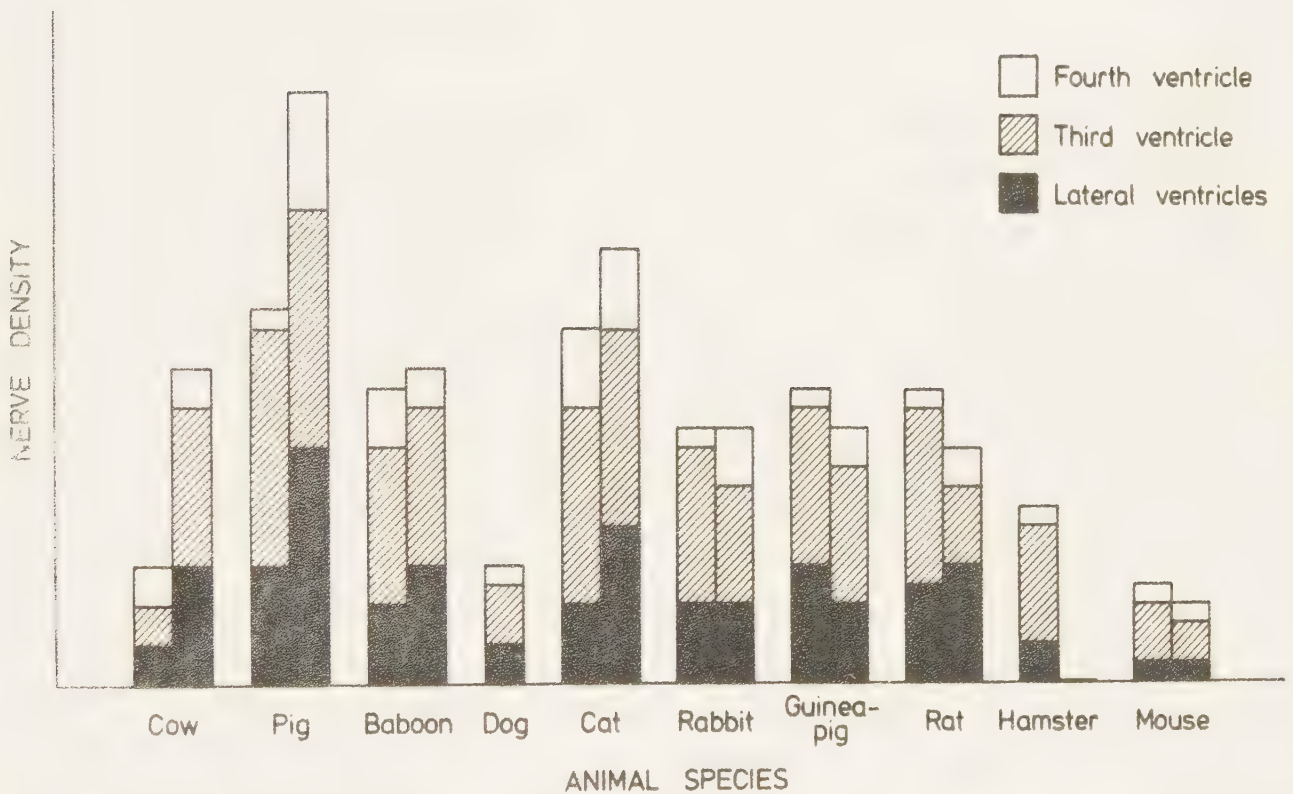
2.3. Histochemistry of cholinergic nerves

The specificity of the commonly used histochemical method for studying cholinergic nerves is limited because it is an indirect method. The technique is based on the assumption that the enzyme acetylcholinesterase is a marker of cholinergic nerves, if pseudocholinesterase is irreversibly inhibited by specific antagonists and the incubation time with acetylthiocholine is maintained short enough to avoid reaction between any residual pseudocholinesterase and the substrate (Holmstedt, 1957; Koelle, 1963). The cholinesterase technique was applied to the choroid plexus of cat and rabbit in preliminary studies by Edvinsson et al. (1973).

Cholinergic nerves (e.g. fibre structures with specific acetylcholinesterase activity) could be demonstrated in choroid plexuses from pig, cat, baboon, cow, rabbit, guinea-pig, rat, dog and mouse, and they thus appear to be a common feature in mammals (Paper III). It was not possible to visualize any nerves in hamster plexuses. If such were present they might have been masked by the specific cholinesterase present in the vessel wall itself (Hardebo et al., 1976), or the method might have been insufficiently sensitive to show delicate nerves with low enzyme activity. The distribution pattern of cholinergic nerves (Edvinsson et al., 1973; Paper III) was the same as for adrenergic nerves (see section 2.2): the cholinesterase-positive nerves either formed networks surrounding blood vessels or ran as isolated fibres in the plexus tufts, where they sometimes were arranged in a wide-meshed network (Edvinsson et al., 1973; Paper III). Pig and cat had the most well-supplied plexuses, baboon and cow, rabbit, guinea-pig and rat had an intermediary nerve density, while dog and mouse did not receive many cholinergic nerves (Paper III). Usually, the plexus from the third ventricle had the richest nerve supply, followed by the lateral ventricles and, as described for the adrenergic nerves (section 2.2), the most sparse innervation was found in the plexus of the fourth ventricle (Paper III). Sympa-

FIG. 1

Relative density of histochemically visualized adrenergic (left bar) and cholinergic (right bar) nerves in the mammalian choroid plexus.



The ventricles from which the plexuses were removed are indicated, together with the 1-unit symbol: arbitrary units from 1 (scattered nerves) to 6 (extensive supply) have been used. The adrenergic nerve supply to the choroid plexus of dog was not examined. The hamster plexuses were devoid of histochemically visible acetylcholinesterase-containing nerves.

thectomy in rabbits and cats, which abolishes all adrenergic nerves at least of the plexuses of the lateral and third ventricles, did not affect the specific acetylcholinesterase activity (Edvinsson et al., 1973; Paper III).

The adrenergic and the cholinergic nerve density in the mammalian choroid plexus is compared in Fig. 1.

2.4. Ultrastructure of the innervation

In an electronmicroscopic study of choroid plexus from rabbit, Millen and Rogers (1956) found large circular profiles, suggested to be portions of autonomic nerve fibres, in the extracellular space and in the basal zone of the plexus epithelium. Twelve years later, Tennyson and Pappas (1968) similarly reported the existence of nerve profiles in the secretory epithelium of the choroid plexus from the fourth ventricle in a human embryo. This was later confirmed in another two embryos, and the structures were thought to be sensory axons (Tennyson, 1975).

Adrenergic nerve terminals are characterized by dense-cored synaptic vesicles (Richardson, 1964), but it is not always possible to separate them from non-adrenergic terminals on this basis by fixation with glutaraldehyde-osmium tetroxide (Hökfelt, 1968). In an electronmicroscopic study of the lateral plexuses from cat and rabbit, the adrenergic nature of the nerve terminals (Paper IV) was secured by the presence of electron-dense synaptic vesicles after injection of the "false adrenergic transmitter", 5-hydroxy-dopamine, which is selectively taken up and accumulated in the synaptic vesicles of adrenergic nerves (Tranzer and Thoenen, 1967).

Thick bundles of axons, enclosed by Schwann cells and a thin collagenous investment, were found in preparations taken near the border of attachment of the plexuses, where they accompanied small arterioles. Further out in the plexus tissue towards the free border, the axons were collected in bundles of smaller size and were located contiguous to the wall of small arterioles as well as to the base of epithelial cells. Near the fringes of the plexus tufts the nerve terminals ran singly and devoid of their neurite-

lemmal sheath (Paper IV).

An approximately equal number of terminals contained electron-dense and electron-lucent synaptic vesicles (30 - 60 nm in diameter) (Paper IV). The absence of 5-hydroxydopamine accumulation in the latter type of terminals indicated that they were non-adrenergic, and against the background of the results at the light microscopic level with the cholinesterase technique (Paper III), it was assumed that they were cholinergic (Paper IV).

Both types of nerves came as close as 20 nm to the membrane of the epithelial cells, where they were located either contiguous to the base of the cell or cellular processes, between adjacent cells, or within a cellular invagination (Paper IV). Similar close relationships have been described in other secretory tissues (Scott and Pease, 1959; Yamauchi and Burnstock, 1967). The distance between the nerve endings and the smooth muscle cells of the arterioles in the plexus tissue was less than 100 nm, which is the distance found in other vascular beds known to receive a true autonomic innervation (Burnstock, 1970), resembling that described for the pial (Hagen and Wittkowski, 1969; Nelson and Rennels, 1970; Nielsen et al., 1971) and intracerebral (Cervós-Navarro and Matakas, 1974) vascular beds. Membrane specializations were absent both at the axonal and muscular side of the adjacent membranes (Paper IV).

Thus, it is concluded that the adrenergic (see section 2.2) and cholinergic (see section 2.3) axons present in the choroid plexus indeed come close enough to the smooth muscle cells of small arterioles as well as the secretory epithelium (Paper IV) to suggest that both components in the plexus receive a true, functioning innervation by autonomic nerve terminals.

3. SYMPATHETIC INFLUENCE ON CHOROID PLEXUS FUNCTION

The histochemical and ultrastructural studies suggested that the innervation of the choroid plexus might influence plexus function through an effect both on its secretory epithelium and on its vascular bed. Separate in-

vestigations were therefore performed (1) to study the sympathetic effects on one parameter of an epithelial function in the choroid plexus, its carbonic anhydrase activity (Paper IV), and (2) to demonstrate by pharmacological methods the presence of adrenergic receptors in the plexus blood vessels (Paper V).

3.1. Sympathetic effect on carbonic anhydrase activity

Carbonic anhydrase has been demonstrated in the choroid plexus epithelium (Giacobini, 1962; Lönnerholm, 1975). This enzyme is considered essential for the production of CSF, and its inhibition with the carbonic anhydrase inhibitor, acetazolamide (Diamox), markedly decreases the production of CSF (for reviews, see Cserr, 1975; Pollay, 1975).

Quantitative determinations of carbonic anhydrase activity were made on homogenates of saline-perfused, blood-free choroid plexuses from the lateral and fourth ventricles of rabbits by measuring the formation of $^{14}\text{CO}_2$ from $\text{NaH}^{14}\text{CO}_3$ in a plexus homogenate (Paper IV). The carbonic anhydrase activity (expressed as $\text{nmol CO}_2/\text{mg}/\text{min}$) was normally twice as high in the plexus of the fourth ventricle compared to those of the lateral ventricles. As expected, treatment of the animals with acetazolamide caused a pronounced reduction in the enzyme activity of both plexus regions. Intraperitoneal injection of reserpine, which depletes the adrenergic nerves of their NA, raised the enzyme activity particularly in the plexuses from the lateral ventricles (Paper IV), probably because the adrenergic nerve density has turned out to be richer there (see section 2.2). The effect of cervical sympathectomy, which was only tested on plexuses from the lateral ventricles, increased the enzyme activity by 150 % at 6 days after the operation, while the activity after 14 days had begun to normalize (Paper IV). This might be due to the progressive development of denervation supersensitivity (Langer et al., 1967) of the adrenergic receptors to circulating catecholamines.

The results indicated that the sympathetic innervation has an inhibitory action on carbonic anhydrase activity in the plexus epithelium and thus,

probably, also on the CSF production.

3.2. Prerequisites for a local sympathetic nerve influence on plexus blood flow

It was suggested by Pappenheimer (as cited by Ames et al., 1965) that blood flow through the choroid plexus might influence the secretory rate from this structure by imposing an upper limit, and the possibility thus exists that at least some of the factors that affect the secretory rate do so by altering blood flow (Cserr, 1971). Choroidal blood flow has in several studies been reported to be relatively high, with values ranging between 200 and 300 ml/100 g/min (Welch, 1963; Pollay et al., 1972; Alm and Bill, 1973, Haywood and Vogh, 1979). These figures are about six times those given for whole brain (Alm and Bill, 1973; Haywood and Vogh, 1979).

In spite of a great interest in mechanisms influencing and controlling CSF formation, little research has been concerned with basic properties of the vascular smooth muscle with regard to the possible involvement of the local blood circulation in the regulation of CSF formation.

A. Definition and characterization of autonomic receptors. Our knowledge about the mechanisms for the interaction between drugs and receptors, as an operational approach to characterize various amine receptor mechanisms, is to a large extent based on the fundamental studies by Furchgott (see e.g. review from 1972). A drug-receptor system is composed of the tissue component that interacts with the agonist, and the complete sequence of events that connect the receptor with the response mechanism. The way to identify the presence of receptor sites is either to obtain responses to various agonists and compare the potencies, or to show that the response can be antagonized by specific receptor-blocking agents. As long as the true receptors have not yet been isolated and characterized biochemically, we have to depend on the assumption that similarities or differences in their response also reflect similarities or differences in the characteristics of the receptors mediating these responses.

In an in vitro study of the anterior choroidal artery from cow, various vasoactive agents were tested and the types of receptors involved defined (Paper V). Special interest was focused on receptors involved in adrenergic mechanisms. Five millimeters from the proximal part of the anterior choroidal artery was dissected out. The vessel receives a well-developed network of adrenergic nerve fibres as shown with the Falck-Hillarp histofluorescence technique (for details, see Björklund et al., 1972). Two pieces of arteries were mounted as cylinders between two separate systems of L-formed metal prongs in the same 50-ml mantled organ bath containing an artificial CSF buffer solution, and the circular vasomotor activity was registered isometrically and reflected by the electrical output from force-displacement transducers. The vessels were given an initial load of 400 dyn and were allowed to relax to a steady state level (approximately 100 dyn lower) during a 90-min accommodation period (for further methodological considerations, see Paper V and Edvinsson and Owman, 1974).

B. Adrenergic alpha receptors (Paper V). A weak contractile effect was obtained with adrenaline, NA, phenylephrine and isoprenaline, while histamine, prostaglandin F_{2α}, and saturated potassium chloride all produced strong contraction. The order of potency for the amines (histamine not included) — tested when the beta receptors were blocked — was found to be adrenaline > NA > phenylephrine > isoprenaline. The contractile effect of NA was inhibited in a competitive way by phentolamine. The results indicated that the vasoconstriction involved alpha-adrenergic receptors of a similar type as found in the peripheral circulation (Furchgott, 1972), but slightly different from corresponding receptors in feline and human pial arteries (Edvinsson and Owman, 1974; Edvinsson et al., 1976).

C. Adrenergic beta receptors (Paper V). Before testing the relaxing effects, the contractile adrenoceptors were irreversibly blocked by pre-exposure of the vessels in the bath with phenoxybenzamine. The vascular smooth muscle is almost completely relaxed under the in vitro conditions used, and an active load

was therefore induced by administration of prostaglandin $F_{2\alpha}$ into the organ bath. The potency rank for the sympathomimetic agents was found to be isoprenaline > NA > adrenaline > terbutaline, and the competitive beta-blocking agent, propranolol, shifted the dose-response curve for isoprenaline in a parallel manner towards higher concentrations. Thus, the relaxation appears to be mediated by beta-adrenergic receptors. As evidenced from the potency relationship between NA and adrenaline, on one hand, and isoprenaline and terbutaline, on the other, the receptors would seem to be of the beta₂ subtype, although further tests with selective agents would be needed for a more conclusive proof. It should be recalled that the relaxing beta receptor in the conventional pial vessels has been classified as a beta₁-type of receptor (Edvinsson and Owman, 1974; Sercombe et al., 1977).

D. Blood flow in the choroid plexus. In view of the low amount of vasoconstriction produced by NA in vitro (Paper V), it is possible that the major part of the sympathomimetic reduction in CSF formation in vivo (see below) is due to direct inhibition of CSF formation at the level of the secretory epithelium rather than a decreased local blood perfusion of the tissue, although the prerequisites for a local sympathetic nerve influence on blood flow exist (Papers IV and V). In fact, Alm and Bill (1973) found no statistically significant reduction in choroid plexus blood flow of the cat when the cervical sympathetic chain was stimulated, and only a weak effect of sympathetic nerve stimulation on rabbit choroid plexus blood flow (A. Bill, personal communication). Moreover, there appears to be no clear correlation between changes in plexus hemodynamics and rate of CSF production. For example, studies with the ventriculocisternal perfusion technique on cat, dog and rabbit failed to associate hypercapnia, of sufficiently high degree to alter cerebral blood flow, with a significant change in CSF production (Hochwald, 1973; Oppelt et al., 1963; Davson and Segal, 1970). Martins et al. (1976) measured cerebral blood flow during ventriculocisternal perfusion of monkey and found that variations of $PaCO_2$ within wide physiological limits has little effect on CSF formation. In a study on the effect of systemic hypo-

tension on the rate of CSF formation in dogs (Carey and Vela, 1974), the amount of reduction in CSF formation could not be correlated with either the fall in mean arterial blood pressure or the reduction in blood volume. Since the CSF formation was lowered only at a very marked hemorrhagic hypotension it was suggested that the CSF formation rate diminished when the blood flow to the choroid plexus or other secretory sites became sufficiently impaired (Carey and Vela, 1974). Vates et al. (1964) suggested the decreasing effect of ventriculocisternal NA perfusion on CSF production in cats to be vascular, while Fishman (1959) recorded no alteration in the rate of sodium exchange between plasma and CSF when NA was administered intramuscularly to dogs in doses that caused sustained systemic pressor elevations. In in vitro studies on dog, rabbit and cat choroid plexuses, Macri et al. (1966) demonstrated a decreased choroidal artery flow rate by NA in the perfusate, and vasoconstriction was observed. The NA effect was in agreement with our findings (Paper V).

3.3. Sympathetic nerve-induced reduction of bulk CSF production

From our studies of the sympathetic influence on carbonic anhydrase activity in the choroid plexus (Paper IV; section 3.1.) and the adrenergic receptor studies in plexus vessels (Paper V; section 3.2.) it is evident that prerequisites exist for a sympathetic nervous influence both on the secretory epithelium and on the blood flow through the plexus tissue, with a consequent net effect on the rate of CSF formation. In order to obtain a direct measure of a possible sympathetic influence on this rate, the ventriculocisternal perfusion technique of Pappenheimer et al. (1962) was applied, largely according to the modifications of Aquilonius and Winbladh (1972), utilizing ^{14}C -labeled inulin (Heisey et al., 1962). The rate of bulk CSF production was determined on the basis of the radioactivity dilution.

The measurements were carried out on rabbits (Paper VI - VIII). The animal was anesthetized, tracheotomized and artificially ventilated, resting prone in a specially made hammock with its head fixed in a metal frame. Artificial CSF, containing inulin- ^{14}C -carboxylic acid (1 μCi /100 ml) was infused through an inflow probe into the right lateral ventricle and drained

at the same rate (30 $\mu\text{l}/\text{min}$) through a needle placed in the cisterna magna, the outflow being collected at 5-min intervals. Infusion pressure was continuously recorded on a Grass Polygraph via the inflow cannula and a Statham pressure transducer. Arterial blood gases were measured frequently by the Astrup technique and adjusted within narrow limits throughout the experiment. Systemic blood pressure was monitored on the Grass Polygraph via a Statham pressure transducer. Body temperature of the animal was maintained normal with a heating blanket and was checked by a rectal thermometer. Radioactivity of the effluent (150 $\mu\text{l}/\text{sample}$) and of samples from the infusate was measured by liquid scintillation spectrometry (quench corrections were made according to the external standard channels ratio method).

Dilution of the ^{14}C -inulin concentration at steady state within the perfused ventricular system was used to calculate the rate at which nascent (inulin-free) CSF was added to the ventricles (Heisey et al., 1962; for details, see Paper VII). For the CSF production measurements to be valid, the diffusional or molecular loss of the marker material from the perfusion fluid has to be negligible. It has been shown by Rall et al. (1962) that there is an entry of inulin through the ventricular system to the brain parenchyma during ventriculocisternal perfusion, but it has been argued in several reports that this escape is insignificant (Heisey et al., 1962; Davson et al., 1962). The molecular weight of inulin is about 5×10^3 , and it has been claimed that the absolute rates of CSF production are more accurately determined by dextran 2000 (mol. weight = 2×10^6) dilution (Davson and Segal, 1970) or using ^{131}I -labeled albumin (mol. weight = 69×10^3) (Curran et al., 1970), thus involving markers of larger molecular size. However, in our studies the primary aim was not to determine the absolute production rates, but rather to compare drug or nerve induced changes in the formation with individual control rates (Papers VI, VII, VIII and IX) or the relative difference in CSF production between two groups of animals (Paper VI). Under these conditions, and as long as any marker escape remains constant, it is assumed that a small loss of the inulin molecule can be neglected.

In one series of experiments the CSF production rate was determined during electric stimulation of the sympathetic trunks in the neck (Paper VI). Stimulation during 1 - 2 hrs reduced the rate of CSF formation by a mean

of 32 % compared to the control situation before stimulation. After cessation of stimulation, the production rate returned approximately halfway towards normal during a 1-hour observation period (Paper VI).

In another series of rabbits, bilateral excision of the superior cervical ganglia (which produces extensive sympathetic denervation of the choroid plexuses) resulted in a 33 % increase in bulk CSF production as compared to an unoperated control group (Paper VI). The results are in agreement with the enhanced carbonic anhydrase activity of the plexus following sympathectomy (Paper IV).

In view of the finding that the sympathetic nerve terminals in the choroid plexus have a close relation to both the secretory epithelium and the smooth muscle cells of the arterioles, indicating a true innervation of both components (Paper IV; section 2.4), the effects of sympathetic nerve stimulation or denervation could have resulted from a direct action on the secretory cells, changes in plexus blood flow through a vascular response, or both. Against the background of the results and considerations presented above in sections 3.1. and 3.2.D, there is reason to believe that the sympathetic action on the rate of CSF production is a combined secretory and vascular effect in the choroid plexus, the former probably predominating.

3.4. Adrenergic receptor mechanisms mediating sympathetic effects on the rate of CSF production

Some of the conditions valid in the pharmacological classification of adrenoceptors were mentioned in section 3.2.A. Possibilities to control experimental conditions are particularly optimal when using small isolated tissues. In the intact animal, however, there are a great number of experimental variables, of which some are difficult or impossible to control. Furchgott (1972) has pointed out some of these important factors, such as the distribution and disposition patterns in the body of the administered drugs, the occurrence of responses which are the resultant of actions of a single drug on more than one kind of organ or tissue, and the modification of the response of a single organ or tissue as a result of nervous reflexes

or humoral feed-back mechanisms. Despite disadvantages with in vivo testing, experiments on intact animals are of great value in receptor studies of complex tissues consisting of, e.g., several components like the choroid plexus, as long as one is aware of existing difficulties, and serious attempts are made to control physiological parameters within narrow limits.

The adrenergic receptors mediating the nerve-induced sympathetic effects presented in section 3.3. were studied by analysing the quantitative changes in the CSF production rate in the presence of various doses of different agonists, with or without specific antagonists (Paper VII). The drugs were either administered in the ventriculocisternal perfusion system or intravenously, with the intention to sort out systemic side-effects and to interpret results with regard to the involvement of epithelial or vascular receptors in the plexus tissue.

Using the ventriculocisternal perfusion technique on cats it has previously been found that the CSF formation rate can be reduced by 24 % and 47 % with NA at a concentration of 2.5×10^{-6} M and 2.5×10^{-4} M, respectively, in the perfusate (Vates et al., 1964). In agreement with this, intraventricular perfusion of NA (10^{-11} - 10^{-3} M) in increasing doses caused a dose-related reduction in the CSF production (Paper VII). The log dose-response curve seemed to comprise two phases with a transition around 10^{-7} M. A maximal inhibition was seen at a concentration of 10^{-3} M. The NA-induced reduction in CSF formation rate was counteracted both by the alpha-receptor antagonist, phentolamine (10^{-6} M) and the beta-inhibitor, propranolol (10^{-6} M); in the presence of the latter antagonist, production was even increased (Paper VII).

The results of the blocking experiments suggested that both alpha- and beta-adrenergic receptors were involved. Of the two components in the dose-response curve for NA, it is possible that the fall in the CSF production seen at low concentrations primarily represents a beta-receptor mediated effect on the secretory epithelium, while that occurring at higher concentrations reflects an added alpha-receptor mediated local vasoconstriction (cf. Paper V), reducing the plexus blood flow.

Also i.v. infusions of NA (5.5 - 55 $\mu\text{g/kg/min}$) gave a dose-dependent reduction in the rate of CSF formation (Paper VII) but, as expected, systemic side-effects were marked. Phentolamine, whether given intraventricularly (10^{-6} M) or i.v. (1 mg/kg), as well as intraventricular perfusion of propranolol (10^{-6} M), counteracted the NA-induced decrease in the production, though the effect of propranolol was weak. On the other hand, there was a potentiation of the reducing NA effect when propranolol was injected i.v. (1 mg/kg). Antagonist doses i.v. were repeated every 45 min.

In an attempt to differentiate that component of the inhibition of CSF formation mediated by the epithelial beta-receptors from the vascular alpha-effect, the ventricular system was perfused with the specific beta-receptor stimulating drugs, H80/62 and terbutaline (Paper VII). Compound H80/62 is a drug which primarily activates the β_1 -type of receptors (Carlsson et al., 1977), while terbutaline mainly stimulates the β_2 -type of receptors (Persson and Olsson, 1970). The CSF production was reduced in a dose-dependent manner by H80/62, while terbutaline had no significant effect. I.v. infusion of either H80/62 or terbutaline had little or no effect on the production rate. Thus, the beta-receptor induced decrease of the production was presumed to represent a direct inhibitory action on the plexus epithelium, primarily supplied with the beta₁ subtype of receptors.

3.5. Involvement of monoamine oxidase in the choroid plexus

During the ventriculocisternal perfusions of sympathomimetic agonists in the rabbits it was found that the effect of NA (10^{-4} M) on the CSF formation was enhanced compared to controls following local inhibition of monoamine oxidase (MAO) by administration of nialamide via the perfusate (Papers VII and VIII).

Considerable MAO activity was found histochemically (Glenner et al., 1957) in the choroid plexuses from all four ventricles of rabbit and mouse, particularly in the cytoplasm of the epithelial cells (Paper VIII; Lindvall et al., 1979b). This agreed with the MAO activi-

ty shown in rabbit, rat, and mouse choroid plexuses by biochemical radio-enzymatic assay according to the method described by Fonnum (1976) (Paper VIII; Lindvall et al., 1979b). Intraventricular perfusion of nialamide reduced the biochemically measured enzyme activity to one tenth of the control value (Paper VIII).

Non-selective inhibition of MAO by nialamide through intraventricular perfusion reduced in itself the CSF formation rate by 44 % (Paper VIII). After sympathectomy (bilateral removal of the superior cervical sympathetic ganglia) the effect of nialamide on the CSF formation was lowered to 22 % (Paper VIII). Administration of 10^{-4} M NA (without nialamide) reduced the CSF production rate by 36 %, representing a near maximum response in the dose-related reduction (Paper VII). After pretreatment with and in the presence of nialamide the production rate was lowered by 65 % in the presence of 10^{-4} M NA compared to the control situation, which is beyond the effect of either NA or nialamide alone (Paper VIII).

Inhibitory experiments with the MAO A inhibitor, clorgyline (Johnston, 1968), and the MAO B inhibitor, deprenyl (Knoll and Magyar, 1972) in vitro (Paper VIII) indicated that the MAO activity in the plexus tissue is primarily of the MAO B type, although MAO A is probably also present in the adrenergic nerves (Goridis and Neff, 1971a and b) of the plexus. This is supported by the above-mentioned finding that sympathectomy reduced the inhibitory effect of nialamide on the CSF production rate. In addition, the effect of sympathetic denervation on the nialamide-induced reduction of the CSF formation indicates that this is under the influence of a substantial inhibitory sympathetic tone under the prevailing steady-state conditions. Some of the MAO activity in the choroid plexus may be involved also in the breakdown of intraventricular NA (Ziegler et al., 1976), which is actively taken up by the plexus, probably into its epithelium (Tochino and Schanker, 1965). However, NA is not a preferred substrate for MAO B (Neff and Yang, 1974). The effect of nialamide that remained in the sympathectomized animals may hence also be the result of — besides any unspecific inhibition of the secretion by the drug itself — an impaired breakdown by MAO (or some other nialamide-

-sensitive degrading enzyme) of other compounds, locally formed in the plexus epithelium or taken up from the CSF, having an inhibitory effect on its formation.

Besides MAO, the choroid plexus of rabbit and rat showed biochemically measurable activities also of catechol-O-methyltransferase (COMT) (Paper VIII; Lindvall et al., 1979b), another enzyme active in extraneuronal amine breakdown. COMT has recently been demonstrated immunohistochemically in both the ventricular ependyma and in the epithelium of the choroid plexus from the lateral and fourth ventricle of rat, beef and chinchilla (Kaplan et al., 1978). This enzyme may share some of the above-mentioned functions of MAO. It is even possible that MAO and COMT, together with the tight junctions between the plexus epithelial cells (see Brightman, 1975), are involved in a gate mechanism for circulating amines that have passed beyond the fenestrated endothelial lining of the plexus.

4. CHOLINERGIC INFLUENCE ON CSF FORMATION

Using the cholinesterase histochemical technique it was demonstrated that the choroid plexus is supplied with cholinergic nerves (Edvinsson et al., 1973; Paper III). Electron microscopy showed close contacts of non-adrenergic nerve terminals, probably identical with the histochemically visualized cholinergic fibres, both with the secretory epithelium and the smooth musculature of small arterioles in the plexus, indicating a true innervation (Paper IV; section 2.4.).

Intraventricular administration of the parasympathomimetic agonist, carbamylcholine, resulted in a dose-related reduction in the CSF formation by as much as 55 % at a concentration of 10^{-3} M (Paper IX). At 10^{-9} M (the lowest given dose) there was even a tendency to a slightly enhanced production. Infusion of acetylcholine (10^{-5} M) in the presence of the cholinesterase inhibitor, neostigmine (10^{-5}), reduced the formation rate of CSF with about the same amount as an equimolar dose of carbamylcholine.

The cholinergic receptors seemed to be of the muscarinic type, since the effect of carbamylcholine was completely blocked by atropine, while hexamethonium was without effect on the carbamylcholine response (Paper IX).

The various test drugs (Paper IX) were administered via the ventricular perfusion system in order to obtain an as far as possible local action without affecting the systemic circulation in a way that might influence the CSF production (Carey and Vela, 1974). It has recently been claimed by Haywood and Vogh (1979) that intravenous carbachol increases the rate of CSF formation in cats. We therefore infused carbamylcholine intravenously into 5 rabbits at rates of 0.025 - 2.5 $\mu\text{g/kg/min}$ during the entire perfusion period (Paper IX). Besides a tendency to a shortlasting increase, carbachol consistently reduced the CSF production rate by 25 - 30 %, in full agreement with the experiments on intraventricular administration. It is possible that the effects reported by Haywood and Vogh (1979) obtained by a single dose injection and recorded only during an ensuing 3-min period, represent the transient initial increment rather than the protracted, and probably more representative, reduction in the formation presently shown during steady-state conditions.

The site of action of the parasympathomimetic agents is still not known. It is possible that the effects mainly involve the secretory epithelium of the choroid plexus rather than its vascular system: carbamylcholine, in contrast to NA, has little or no vasomotor effect on isolated small choroidal arteries (Paper V), and, if anything, it should be expected that the cholinergic agents would increase plexus blood flow and thus not provide a lowered CSF formation.

5. GENERAL SUMMARY

(1) The mammalian choroid plexus receives well-developed systems of both adrenergic nerves (demonstrated by fluorescence histochemistry and radioenzymatic determination of tissue NA) and cholinergic nerves (visualized by acetylcholinesterase staining), showing similar distribution patterns in the tissue. Thus, the nerve terminals not only enclose small blood vessels, but they can also be followed in between the secretory epithelium and the underlying vessel wall in the plexus tufts, sometimes forming a delicate network. The autonomic nerve supply is particularly rich in the third ventricle plexus, followed in order by those of the lateral and fourth ventricles. The adrenergic nerves were in denervation experiments found to originate in the superior cervical sympathetic ganglia.

(2) Developmental studies performed in rabbit showed the first histochemically visible adrenergic nerves to appear in the plexus of the third ventricle within the first day after birth. Nerves became visible shortly afterwards in the lateral plexuses, but not until two weeks post partum in the fourth ventricle plexus. Full development of characteristically varicosed axon terminals were seen in the whole plexus system at three weeks. The pattern of development could be confirmed by radioenzymatic determination of tissue NA.

(3) Electronmicroscopy showed that the autonomic nerves innervate both the vascular smooth musculature and the plexus epithelial cells in a true sense, with neuro-effector membrane distances of 90 and 20 nm, respectively.

(4) A sympathetic inhibitory effect on the plexus epithelium was indicated in determinations of carbonic anhydrase activity. Pharmacological in vitro analysis showed the presence of vasoconstrictory alpha-adrenergic and vasodilatory beta receptors in the choroidal arteries. Thus, the sympathetic nerves may effect the CSF production from the choroid plexus through an action both on its secretory epithelium and the vascular bed.

(5) The bulk formation of CSF was measured in rabbits by the ventriculocisternal perfusion technique of Pappenheimer using ^{14}C -inulin dilution. Electrical

stimulation of the sympathetic trunks in the neck markedly reduced the CSF production, whereas sympathectomy by removal of the superior cervical ganglia had the opposite effect.

(6) Pharmacological experiments in vivo suggested that the sympathetic inhibition of the CSF production rate is the result of a combined beta₁-receptor mediated inhibitory action on the plexus epithelium, and a reduced blood flow in the tissue through constriction via the vascular alpha receptors.

(7) The choroid plexus contains MAO, primarily of the B type, but probably also type A in its sympathetic nerves. Inhibition of the enzyme reduces the CSF production rate, an effect that was abated by sympathectomy. The results suggest that MAO participates in the regulation of an inhibitory tone, in which the sympathetic nerves are involved, on the CSF production.

(8) The CSF production rate is also reduced by cholinomimetic agents, an effect that implicates the presence of the muscarinic type of cholinergic receptors in the choroid plexus.

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